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PRINCIPAL INVESTIGATOR: Harry A. Drabkin, M.D.

CONTRACTING ORGANIZATION: University of Colorado, Denver
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Loss of tumor suppressor genes (TSGs) represent critical molecular events in the development and progression of breast cancer. Based on loss of heterozygosity (LOH) studies as well as direct cytogenetic studies of breast tumors, one or more TSGs likely resides on the short arm of chromosome 3 (3p) and appears to be involved in nearly 50% of breast cancers. Four distinct regions within 3p [p12, p14, p21 (proximal) and p21 (distal)] undergo recurrent deletions in human carcinomas and are the most likely sites for a breast cancer TSG. We demonstrated recurrent homozygous deletion or rearrangement in breast cancer cell lines involving 3p14. A set of DNA clones spanning the critical region has been sequenced revealing several potential gene coding segments, some of which we now know are expressed in breast tumors. YACs containing the critical region have been modified with the Neomycin resistance gene for selective retention in mammalian cells. Introduction of the YAC into a murine fibrosarcoma cell line appears to inhibit its tumorigenicity, supporting the hypothesis that a tumor suppressor gene resides in the critical region. Evaluation of other regions for involvement in breast cancer as originally proposed using deletion detection, DNA sequence analysis, gene isolation and expression assays, is ongoing.

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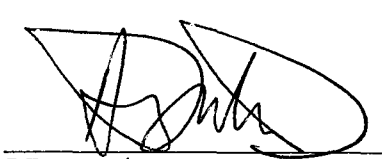

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5. INTRODUCTION

5.A. Purpose of the Present Work

Our project concerns the identification and isolation of a breast cancer tumor suppressor gene from the short arm of chromosome 3 (3p). In the early stages of this investigation, we identified a region of homozygous deletion in a subset of breast cancer cell lines suggesting that a tumor suppressor gene would be found in the region of loss. The deletion occurred in a segment of DNA within a few hundred kilobases (1 kb = 1,000 base pairs, a measure of distance along the DNA molecule) of a chromosomal rearrangement involving chromosomes 3 and 8 which is associated with hereditary kidney carcinoma (Cohen et al., 1979). (It is often the case that a tumor suppressor gene is involved in more than one type of cancer, and both kidney and breast cancers are of epithelial cell origin.) The region surrounding this breakpoint (located in band 3p14.2) was one of the possible target loci described in our original application. In our last Progress Report, we noted that the order of our specific aims had been modified to examine this region in detail first. This allowed us to develop necessary DNA reagents to study the target area thoroughly and to identify potential genes. The progress we detailed in the last report included assembly of completed sequence for over 150 kilobases of DNA and identification of a number of potential gene coding regions (exons). We have now published a first manuscript on these results (Boldog et al., 1997), which is included in the appendix, and have significantly extended them. In particular, sequences have been generated for more of the critical region, some of the putative genes are now known to be expressed in breast tumors and we have demonstrated that the region displays ongoing genetic instability in cancer cells. Importantly, gene replacement experiments are beginning to indicate that an apparent tumor suppressor function lies within the critical region we have sequenced. Our previous report documented some of the efforts we were directing at three additional deletion sites which may harbor important breast cancer related genes. We have now uncovered a new gene in proximal 3p21 and developed a set of contiguous PAC clones covering the most distal homozygous deletion region in 3p21. Distal and proximal refer to directions along the chromosome, with distal meaning towards the telomere, the structure which caps the chromosome arm and proximal meaning towards the centromere. The current Progress Report will first review our progress in the 3p14 homozygous deletion region and then cover new findings in these other regions.

5.B Nature of the Problem

Basic concepts especially for the lay reader We realize that the scientific literature is nearly totally composed of technical terms. Throughout this report we have attempted to explain these concepts in lay terms.

The malignant potential of any tumor, including breast cancer, is a consequence of specific alterations (mutations, deletions, amplifications, etc.) in target genes that regulate the growth and biologic behavior of those cells. (Genes are segments of DNA which encode proteins; DNA is "transcribed" into RNA and RNA in turn is "translated" into protein.) Whether cells grow slowly and remain localized, or proliferate rapidly and spread to distant sites (metastasize) is a complex process involving a host of regulatory genes. For example, loss or mutation of the p53 tumor suppressor gene, located on 17p, is associated with instability of the genome (entire DNA of the cell) (Yin et al., 1992; Livingstone et al., 1992) and a worsened prognosis. This instability results in an enhanced capacity of the malignant cell to undergo DNA rearrangements leading to alterations in critical regulatory genes. Loss of normal p53 function is also associated with the cell's ability to escape death or cell cycle arrest resulting from therapeutic radiation or chemotherapy (Lowe et al., 1993).

In other instances, the critical regulatory genes have yet to be identified. This is the case for genes located on 3p although we now have candidates in target regions. Scientific investigations

have provided strong evidence pointing to where certain types of critical genes are likely to be located. For example, cytogenetic studies, which examine the content and nature of chromosomes within cells, have identified certain recurrent abnormalities in cancers. Specific chromosomal segments have been found to be increased in number (amplified). This finding is expected to be associated with overexpression of a gene (because of its increased copy number). Such genes, for example, may encode growth factor receptors or may encode proteins that mediate resistance to chemotherapeutic agents. An example is provided by the MDM2 gene whose protein product inhibits the activity of p53; overexpression of MDM2 is thought to have consequences similar to mutation in p53. In contrast, cytogenetic studies have also pointed to recurrent deletions involving specific chromosomal regions. The critical genes believed to be encoded in these regions are referred to as tumor suppressor genes, the type of gene located on 3p which is the focus of our investigation.

The nature of known tumor suppressor genes is quite varied. Certain tumor suppressor genes, e.g. p16 (an inhibitor of the cyclin dependent kinases or CDKs) and the retinoblastoma gene (RB1, an inhibitor of the E2F transcription factor), control cell division by regulating the process of DNA replication. Some tumor suppressor genes, such as the chromosome 18 gene DCC (Deleted in Colon Carcinoma), are located on the cell surface. Interestingly, as this may relate to our studies on the Semaphorin IV gene located in the proximal 3p21.31 deletion region, DCC has recently been shown to encode a receptor for a netrin, a protein involved in nerve growth cone development (Keino-Masu et al., 1996). Although a mouse mutation which destroys netrin function does not generate murine cancers (Fazeli et al., 1997), this may result from differences between mice and humans. This demonstrates that molecules initially identified in signaling pathways associated with nerve growth cone guidance may be involved in cancer. A common feature in this class of tumor suppressor genes is that their normal function is lost as part of tumor development. As a consequence, a regulatory function generally affecting growth and differentiation is also lost. The resulting cell may divide more frequently than is appropriate, giving rise to a clone or small cluster of related but abnormal cells and setting the stage for further genetic changes.

5.C. Methods to Isolate/Identify Tumor Suppressor Genes

5.C.1 Positional Cloning.

Tumor suppressor genes have been isolated by two approaches. Perhaps the most frequently used method is referred to as "positional cloning" in which the region of chromosomal loss is defined by molecular (DNA) probes and cytogenetic analysis. Because a visible chromosome deletion represents a large expanse of actual DNA, it is necessary to narrow the target region as much as possible. In one approach, this is done by using "polymorphic" probes which can distinguish between the two copies of the chromosome in question (each being inherited from one parent). In the tumor DNA, loss of one copy (by a variety of mechanisms) is referred to as "loss of heterozygosity" (abbreviated LOH). To detect LOH, a DNA probe must exist which corresponds to the target DNA in question and, importantly, this bit of DNA must exhibit a frequent, naturally occurring, variation in the population. Such variations can usually be detected experimentally and they provide a means to identify differences between the two chromosome copies in any individual tumor sample. Naturally occurring differences in DNA sequence are not uncommon although some types of DNA sequences, referred to as "microsatellites", exhibit much more variation than other types. Microsatellites consist of repeated pairs of nucleotides (usually cytosine followed by adenosine, abbreviated CA) at specific chromosomal sites with the important feature that the number of pairs is variable from person to person. Since the natural variation of markers is often a limiting factor even with microsatellite markers, this explains the general necessity to test fairly large numbers of tumor samples with different DNA probes. The goals of LOH experiments are to identify one or more target regions and to narrow such regions as much as possible prior to performing gene searches.

5.C.2 Candidate Genes.

A second approach in identifying tumor suppressor genes is through the testing of candidate genes. These candidates come from two sources; first, genes mapped within regions of recurrent deletion (which is essentially the positional cloning approach except that the genes have already been identified) are all considered candidates until proven otherwise, and second, those candidates whose known function suggests they might be targets independent of chromosomal position. While our studies continue to involve the positional cloning approach, a number of candidate loci have now been identified in the target regions and we are beginning to test these. These genes include several putative coding regions within 3p14.2, the semaphorin IV and DEF-3 genes in 3p21.31 and at least 5 cDNAs identified in 3p21.33.

5.C.3 Identification of Homozygous Deletions Greatly Facilitates Positional Cloning of Tumor Suppress Genes

LOH studies can lead to the identification of homozygous deletions in tumors, an extremely important finding which can greatly facilitate the precise positioning of tumor suppressor genes. Furthermore, homozygous deletions provide one of the best ways to chose particular candidate genes from a larger set, based upon their location within the minimal deletion defined experimentally. These often rare events thus provide a powerful adjunct to LOH and candidate gene studies.

A homozygous deletion means that both copies of a chromosome have undergone loss for a particular segment of DNA (see diagram below).

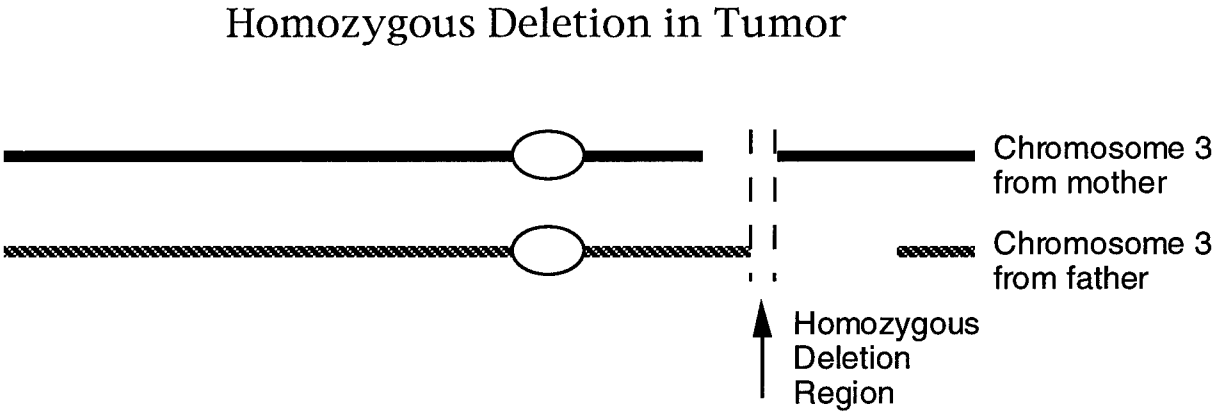


Figure 1. Diagram of chromosome 3 homologues with overlapping deletions in a tumor.

Such deletions represent one of several mechanisms that can lead to the complete loss of a tumor suppressor gene. While tumors frequently lose one tumor suppressor gene copy by undergoing large chromosomal deletions, the remaining copy is usually rendered non-functional by other genetic mechanisms. These can include point mutations and gene silencing (caused by hypermethylation) as well as a second deletion leaving a complete gap in the genetic material.

5.C. Additional Background Information.

In this section, we present a summary of data from the literature regarding genetic deletions of chromosome 3 in breast cancer. These selected studies relate both to our identification of a homozygous deletion in 3p14 as well as genetic changes in other regions. We have placed these comments here for the convenience of the reader, since the concepts of loss of heterozygosity (LOH) and homozygous deletions have been introduced earlier.

Sato et al. (Sato et al., 1991) examined 120 breast cancers for loss of heterozygosity (LOH) using a series of 3p polymorphic loci. Nearly 50% of the informative tumors (56/120) demonstrated LOH involving 3p. In this study, the 3p region undergoing the greatest loss was 3p14.2-p13 which should include the homozygous deletion region we have identified. This study has been one of the largest in terms of the number of tumors examined and 3p-derived probes tested. One significant limitation was that the type of probe used did not detect sufficient polymorphisms to allow the limits of the 3p target region to be defined more precisely. Another study using LOH analysis demonstrated that 3p loss was most frequently seen in familial breast cancers (Bergthorsson et al., 1995). Excitingly, the highest frequency of loss (68%) was with a probe (D3S1217) also located in the 3p14 region. A cytogenetic study (chromosome analysis by direct microscopic visualization) by Pandis et al. (Pandis et al., 1993) identified 3p deletions in 5 of 41 breast carcinomas. Importantly, the deletions appeared nearly identical and involved the 3p13(p14) region implicated above using LOH analysis. Intriguingly, in the study of Pandis et al., in 3 of the 5 cases the 3p deletion was seen as the only recognizable cytogenetic abnormality. This suggests that 3p deletion may be an early event in a subgroup of breast cancers. Taken together, there is considerable evidence from multiple investigators using different techniques which supports the frequent alteration of 3p14 during breast cancer development.

Additional data from LOH and cytogenetics studies strongly implicate several other 3p regions that may harbor genes important in breast cancer. Chen et al (Chen et al., 1994) found LOH within 3p13-14, 3p21-22 and 3p24-26 along with one case of homozygous deletion in 3p13 likely corresponding to our homozygous deletion. All three of these target sites have been previously implicated in other carcinomas, particularly of lung. Hainsworth et al., (Hainsworth et al., 1991) found frequent chromosomal breakpoints at 3p21 which can be indicative of gene disruption or deletion. In a study by Ali et al, (Ali et al., 1989), the shortest region of common loss was between the markers D3F15S2 and RAF1, spanning the chromosomal region 3p21-p25 and a similar study by Devilee et al (Devilee et al., 1989) found the p14 to p21 region commonly deleted. Several other studies corroborate these findings having observed interstitial 3p deletions (Teixeira et al., 1996) or other structural changes affecting this chromosome (Lu et al., 1993). Intriguingly, Buchagen et al., (Buchhagen et al., 1994) identified one apparent rearrangement and one homozygous deletion for the probe D3S2, known to map within proximal 3p21.1 (Gemmill et al., 1991) very close to the semaphorin IV deletion region (Roche et al., 1996). These studies all implicate multiple target regions on 3p which appear to be very similar to regions identified from studies on carcinomas derived from other tissues. Based on these studies, we have focused efforts on three homozygous deletion regions. Our investigation of 3p14 has matured; we are now conducting transfection experiments to test for functional tumor suppression as well as pursuing several novel genes which are present in the region. In addition, we have demonstrated that 3p14 is inherently unstable in tumor cells with p53 mutations, and appears to be particularly susceptible to genetic losses. We are also carefully examining two candidate genes in proximal 3p21 and have completed development of a contiguous set of clones covering an estimated 750 kb of the distal deletion region in 3p21.

5.D. Review of Specific Aims

5.D.1. Our original Specific Aims were designed to:

1. Define the regions of 3p undergoing LOH in breast cancer.
2. Test known 3p candidate genes for mutations in breast tumors.
3. Isolate additional candidate tumor suppressor genes from regions we define.
4. Characterize the product(s) of these genes and assess their involvement in breast cancer.

In the last two Progress Reports, we described how these aims were modified to reflect the substantial effort being focused at that time on the recurrent homozygous deletion region involving 3p14. In the last Progress Report, we noted that many of the previous year's goals had been

achieved and we provided a detailed account of the results. A manuscript describing our cloning of FRA3B, discovery of homozygous deletions in the region and sequence analysis has now been published (Boldog et al., 1997). In addition, we noted that the progress made on 3p14 allowed us to pursue several other sites where potential breast cancer tumor suppressor genes might be found, following the original strategy and aims of our proposal. Below, we will summarize the published progress and then provide details of accomplishments in the past year. These include testing breast cancer lines for deletions in several regions, examining putative genes in 3p14 for expression and testing the 3p14 region for tumor suppressor function. Our efforts to understand the potential role for genes in the proximal 3p21 region have progressed significantly and we have developed the necessary clone contig to begin a detailed examination of the distal 3p21 deletion region.

6. BODY

6.A. Summary of previous findings.

6.A.1. Examination of breast cancer cell line DNAs for homozygous deletions in 3p14.

As mentioned, one of the most frequently deleted regions in breast cancer is 3p14 (Yokota et al., 1989). Southern blot hybridizations detected homozygous deletions or rearrangements with probes from 3p14 and DNAs from breast cancer cell lines. Thirteen breast carcinoma cell lines utilized were: CRL1504, HTB122, HTB23, HTB121, HTB123, HTB127, HTB131, HTB132, MCF7, MDA231, T47DV, ZR751 and HTB126. MDA231 contained a discontinuous deletion while CRL1504 and HTB123 contained apparently rearranged bands. We developed a ~300 kb cosmid/lambda (DNA clone) contig (Fig. 2) in the region of the homozygous deletions (Boldog et al., 1997). From sequence and hybridization data, we showed that the cloned region corresponds to the most inducible common fragile site in the genome, FRA3B (Glover et al., 1984). Fragile sites are DNA regions which are unstable. In a few cases of rare fragile sites, their nature has been elucidated at the DNA sequence level and appears to be due, at least in part, to an expanded triplet repeat which may interfere with normal DNA replication. Our results clearly demonstrated that FRA3B represents a region rather than a single site. Where we have accurately defined the boundaries for the carcinoma-associated deletions, one or both boundaries are contained within the FRA3B region showing that these deletions overlap FRA3B.

During our studies, Ohta et al. (Ohta et al., 1996) identified the FHIT gene with reported abnormalities in RT-PCR products involving a variety of carcinoma cell lines, especially colorectal and lung carcinomas. These and other investigators also demonstrated abnormalities in breast cancer cell lines. (In RT-PCR experiments, RNA is isolated and reverse transcribed into cDNA. PCR primers are then used to greatly amplify this product using the polymerase chain reaction which can then be examined for its correct size or its DNA sequence can be determined.) While FHIT could be the putative tumor suppressor gene, we think that it is not for many reasons (Boldog et al., 1997). First, the smallest deletion we identified (in cell line CC19) does not involve FHIT coding sequences and from our RT-PCR and cDNA sequence analysis the coding portion of the FHIT transcript is normal in this cell line (Boldog et al., 1997). This excludes the possibility of an undetected mutation in the coding portion of FHIT. Second, we observed that FHIT undergoes alternative splicing in normal tissues (Boldog et al., 1997) which explained some previously reported abnormal PCR products (Ohta et al., 1996; Sozzi et al., 1996; Virgilio et al., 1996). Our hypothesis was supported by the report of Thiagalingam et al. (Thiagalingam et al., 1996) who observed the lack of FHIT involvement in colorectal carcinomas and suggested that PCR artifacts might be responsible for some observed alterations (Ohta et al., 1996; Sozzi et al., 1996). Third, discontinuous deletions, as we observed in the breast cancer cell line MDA231, appeared common in this region, both from our analysis and from that reported by Ohta et al. (Ohta et al., 1996). Discontinuous deletions might be expected if they occurred in a region of underlying genomic instability, whether or not they were being biologically selected for. Fourth, FHIT has similarity to yeast di-adenosine hydrolase (a metabolic enzyme) which would represent an unexpected function

for a tumor suppressor gene. Fifth, and very importantly, re-introduction of FHIT into cell lines where it was altered has had very little consequence (Dr. Carlo Croce, personal communication). Sixth, one of the possible features that suggested FHIT could be a tumor suppressor gene was that it crossed the hereditary kidney carcinoma associated 3;8 breakpoint (Ohta et al., 1996). However, we found no alterations in RT-PCR products from 5 renal carcinoma cell lines, and Dr. Gyula Kovacs (Heidelberg, Germany) has observed normal FHIT transcripts with no point mutations in a large series of kidney cancers.

Thus, we are faced with two contrasting, but not mutually exclusive, possibilities. First, another candidate tumor suppressor gene may exist within 3p14 and FRA3B, a possibility we are carefully pursuing. Second, the deletions may be due to primary genomic instability affecting a particularly susceptible region. In fact, we have observed the highest frequency of deletions in cervical carcinomas where p53 inactivation is very common (Milde-Langosch et al., 1995). This suggested that p53 mutations may be necessary for 3p14 instability but not sufficient (Boldog et al., 1997) since many tumors have p53 mutations and only a subset have deletions.

6.A.2 DNA Sequencing and Analysis

We undertook DNA sequencing studies as a means to identify genes and to understand the instability of the region (Boldog et al., 1997). DNA sequencing has been completed for approximately 170 kb and our analysis of the sequence features has been published for a contiguous stretch of 110 kb (Boldog et al., 1997). (The DNA sequence analysis involves computer algorithms to identify all repetitive DNA sequences, identities and similarities to known genes contained in various databases, predicted gene segments and various other structural/compositional features.) We found the region to be very high in A-T content with frequent LINE and MER repetitive elements, and conversely low in Alu repetitive elements and confirmed genes (Fig. 3). In contrast to the reported rare folate-sensitive fragile sites, which are associated with expanded CGG repeats (Pintado et al., 1995; Nancarrow et al., 1995), FRA3B does not contain an expanded triplet repeat or methylated CpG-island. We found a number of potential gene sequences; analysis of these will be described in detail below.

6.A.3. Summary and Significance of 3p14 Findings

The identification of a DNA segment which undergoes recurrent homozygous deletion and rearrangement, as we have observed for this region of 3p14, suggests that it encodes a tumor suppressor gene. However, at the present time our data do not support FHIT as the 3p14.2 tumor suppressor gene. We propose an alternative hypothesis; that genomic instability per se may lead to the observed deletions. There may also be other genes in 3p14 which are tumor suppressors. These are contrasting hypotheses which can only be answered by careful study. We have identified other putative genes in the deletion region, any one of which may be a target for deletions, and our studies will continue in this direction. For a functional assay of tumor suppression, we are introducing YACs from this region back into cell lines that show deletions. Our studies of this region address each of the 4 specific aims. Clearly this is an identified region of loss (aim 1). The analysis of FHIT DNA coding sequences has failed to support its involvement as a tumor suppressor gene (aim 2). Our search for additional genes is very much ongoing (aim 3) and finally, the introduction of YACs from this region (aim 4) is providing independent functional evidence for the presence of a tumor suppressor gene.

(UNPUBLISHED DATA)

6.B.1 Novel Genes in FRA3B and 3p14.

The sequence data (diagrammed in Fig. 3) revealed a number of potential gene encoding regions within FRA3B. Many exons were predicted by GRAIL2 and GeneMark exon prediction

programs (Borodovsky and McIninch, 1993), but nearly all occurred within repetitive elements of the LINE and MER families. Some showed identity with an EST from the dbEST sequence database, but these were discarded as candidate genes because they appeared to be primed from a genomic poly-A tract and were co-linear with genomic DNA. (ESTs are short DNA sequences obtained from the ends of cDNA clones. While most cDNA clones represent parts of genes, there are some which are derived from incompletely spliced RNA and others which may represent DNA contamination in the library.) Of five highly predicted exons which remained, none have shown similarity to known genes. Several exons with lower probability scores occured in regions which were identified by other experimental evidence. Thus, this portion of the deletion region has the potential to encode more genes than FHIT. We have explored two of these so far and will continue this process and extend our analysis to the others during the coming year.

λ 58.

Cleavage sites recognized by rare-cutting restriction endonuceases (REs) such as NotI, SacII, EagI, etc., are associated with CpG islands (regions with high GC base pair content) and genes (Cross and Bird, 1995). The identification and isolation of these rare sites provides a rapid means for isolation of potential genes. One SacII site was identified within contig clone λ58 which was subsequently isolated by island-rescue PCR. Sequence analysis placed this site about 10 kb distal to FHIT exon 5 and close to a GRAIL2 predicted exon (Figs. 2 & 3). Reverse transcriptase - polymerase chain reaction (RT-PCR) experiments confirmed that the λ58 exon cluster was highly expressed in the breast cancer cell line HTB 24 (Fig. 4, lane 1) but a control neighboring PCR marker (GapS, Fig. 4, lane 9) was poorly expressed. Importantly, a YAC transfectant of murine A9 cells also expresses λ58 (lane 5) while the control locus GapS is not expressed at all in these cells (lane 13). This indicates that λ58 is specifically expressed in HTB 24 and from the YAC in A9 cells, even though the products were not detectably different in size from genomic controls. The absence of splicing suggests that the λ58 region analyzed so far comprised the 5' or 3' untranslated region (UTR) or that the gene was intronless. Alternatively, the RNA template may have represented unprocessed primary transcript. We utilized rapid amplification of RNA ends (RACE, (Frohman, 1993)) in both the 5' and 3' directions to clone more transcribed portions both upstream and downstream from the λ58 region. Sequence analysis of 5' RACE clones revealed the direction of transcription (as indicated in Figs. 2 & 3) and extended the sequence in the 5' direction by 120 bp. However, although there are relatively long ORFs in both directions, there are no homologies in the databases and no matching ESTs. Our efforts on λ58 will continue in the next funding period.

The λ58 exon cluster is particularly interesting because of preliminary results, described later in detail (see section 2.B.2, below), suggesting that tumor suppressor function is encoded within the region from which λ58 derives. The YAC clone used for these experiments (y74B2G) includes only exon 5 of FHIT, thereby excluding it as the tumor suppressor in this situation. However, when the YAC is placed into murine A9 cells, λ58 sequences are specifically expressed from the YAC (Fig. 4, compare lanes 3 and 5 with lanes 11 and 13).

GB exon.

The GB exon (Figs. 2 & 3) was identified by exon trapping using the BRL-LifeTechnologies exon trapping kit. Sequence analysis has shown that three exons reside in this region with probabilities of >85%. RACE experiments both 5' and 3' have yielded many products whose sequences are currently being determined. Exploration of this potential gene sequence will continue, to determine if it is expressed and to identify other exonic sequences that may be linked to it. There are a number of additional putative exons shown in Fig. 3 (bars above the line) which need to be explored carefully, as we plan to do.

HRCA1.

Our previous studies demonstrated that the region encompassing the hereditary kidney cancer 3;8 translocation on chromosome 3 contained a gene called HRCA1 (Boldog et al., 1993) (Fig. 2 & 5A). HRCA1 resides within a few kb of the 3;8 breakpoint on its telomeric (or distal) side. Several overlapping cDNA clones (K7, K20, BII and BIII) were isolated but sequence analysis of these clones showed no significant open reading frames that might encode a protein. However, both GRAIL2 and GeneMark predicted high-probability exons and two of these were subsequently trapped (Fig. 5A) using the BRL-LifeTechnologies exon trapping kit. Several additional exons have been trapped from surrounding genomic clones, and one of these proved to be identical to exon 4 of FHIT, definitively placing the HRCA1 exon cluster within the third intron of FHIT.

GeneMark and GRAIL2 predictions together with the exon-trapping results implied that the cDNAs for HRCA1 were generated from incompletely processed message, a finding which was corroborated by subsequent RT-PCR experiments. RNAs isolated from 9 tumor cell lines (3 breast, 2 lung, 2 cervix, 1 colorectal and 1 kidney) were treated with reverse transcriptase (RTase) and PCR amplified using two HRCA1 specific primers (Fig. 5B). Many RNA samples, particularly those from breast tumors, yielded unspliced products (709 bp) which were absent (or greatly reduced) in controls without RTase. In addition, a band of 600 bp was observed when filter transfers were hybridized with an exon specific primer (Fig. 5C), suggesting that splicing occurs within this transcript. Interestingly, this smaller product was observed most strongly in the breast carcinoma cell lines HTB24, MCF7 and HTB121. We are continuing to study HRCA1 expression by both 5' and 3' RACE experiments.

The above results strongly suggest that more genes than FHIT are encoded in the 3p14 homozygous deletion region. These putative genes need to be thoroughly investigated as candidates for tumor suppressors important in breast cancer, as we are doing.

6.B.2 Introduction of YACs into recipient cells.

As a functional assay and as an alternative means to identify potential tumor suppressor genes within large cloned DNA segments, we proposed to introduce YACs into recipient cell lines and then to assay the tumorigenicity of the resulting cells in immunodeficient (nude) mice. This represents specific aim 4 as applied to this chromosomal segment. In our previous reports, we described progress in the introduction of a selectable marker (retrofitting) into YAC 74B2g with pRV1 (neoR vector) and the verification of the correct integration. This YAC, containing approximately 450 kb of human DNA from 3p14, represents a portion of the deleted segment in the breast cancer cell line MDA231. To test the system initially, we chose mouse A9 cells for several reasons. First, other investigators have more frequently succeeded in introducing YACs into rodent cell lines. Second, we could easily examine recipient cells to determine if the YAC had been transferred intact or if it had been disrupted. Third, mouse A9 cells are tumorigenic in nude mice and we could ask whether any genes contained in YAC 74B2g affected this.

In our last report, we had succeeded in transferring the intact YAC into A9 cells. Of the 12 G418 resistant clones obtained, two (clones #10 and #12) were positive for both the left arm marker and the right arm neoR marker. This indicated that in these two clones the YAC was likely intact, which was confirmed by FISH analysis using two cosmid clones (31E1, 55D12) located towards the ends of the human insert in the YAC. Our initial testing of the tumorigenic potential of A9 parental and 74B2g transfected cells in *nude* mice demonstrated that the 74B2g transfectant was highly suppressed in its ability to form tumors compared to A9 parental cells. One half million cells of both A9 and A9-74B2g were injected into 20 and 15 mice, respectively. At three weeks post injection, the A9 cells had grown into obvious tumors of at least 2 cm in diameter while the A9-74B2g transfectants were at most 2 to 3 mm in diameter, if visible at all. We had to sacrifice the A9 parents shortly thereafter due to tumor burden while the A9-74B2g injected mice survived for an additional 4 to 5 weeks. Eventually, the A9-74B2g mice developed tumors which were qualitatively similar in size to the A9 parents, but the rate of growth was substantially slower. We

are repeating these experiments with a transfectant of an additional YAC (850A6) which contains an overlapping section of the 3p14 region. These experiments will allow us to obtain critical quantitative data on tumor size and growth rate. It was possible that the suppression of tumorigenicity observed with A9-74B2g could have been caused by a general decrease in the growth rate of transfected cells due to the introduced YAC . This was tested by measuring the *in vitro* growth rate of both parental control and stably transfected cells. We could discern no difference in their *in vitro* growth rates, as shown in Fig. 6. Interestingly, cervical tumor cells with differing deletions in the FRA3B region also appear to differ remarkably in their tumorigenicity, and this may be due to the same sequences as in the A9-74B2g transfectants.

6.B.3 Ongoing instability of 3p14.

Coincidence of homozygous deletions in 3p14 with FRA3B sequences suggested that the region represented by the fragile site was inherently unstable. Our published observations implied that while p53 mutations were a necessary prerequisite for deletions, they were insufficient by themselves. We reasoned that if the region was inherently unstable, then carcinoma cell lines which still retained much of the region might be progressively deleting more of FRA3B and these events could be measured. Our strategy was to subclone selected cell lines and to analyze their DNA for alterations within FRA3B using PCR and Southern blots. The sequence data provided the high density of markers (17 markers over 170 kb) necessary to detect further deletions. The cervical line CC19 and the breast line MDA231, both with defined deletions (Fig. 2), were subcloned and analyzed. Eleven of twenty five CC19 subclones (44%) had new deletions, no two of which were identical. None of the new deletions were an extension of the original 45 kb deletion characteristic of CC19 but were new losses extending in the telomeric direction. Several of the subclones had multiple, discontinuous new deletions. The breast carcinoma cell line MDA231, in contrast, has shown no evidence for new deletions although all the markers have not yet been tested. These differening results could be due to inherent genetic differences between the two lines, such as the copy number of chromosome 3, or they could reflect a biological difference in the rate of deletions within FRA3B. Nevertheless, these results strongly support our contention that the FRA3B region is inherently unstable and that this instability is ongoing.

6.B.4. Summary and Significance of 3p14.

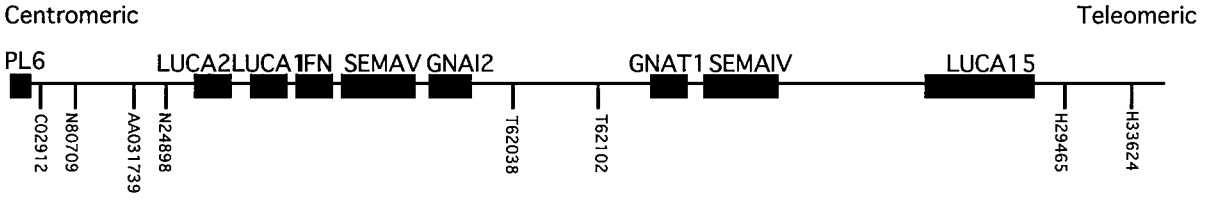
Overall these results suggest that 3p14 is inherently unstable in at least a subset of tumors. In addition, our YAC replacement experiments imply that a functional tumor suppressor resides within the 74B2 sequence and this may correspond to λ58. We are currently in the process of dissecting this region and clearly identifying the sequences responsible for this suppression effect. Although we believe that FHIT is not responsible for suppression (the genetic material contained in 74B2 would corroborate this) we nevertheless plan on introducing a FHIT expression construct to directly test this.

6.C. Involvement of 3p21 in Breast Carcinoma.

6.C.1. Known and Predicted Genes in 3p21.31.

Chromosome band 3p21 has long been thought to contain a tumor suppressor gene based upon two seminal observations. First, the region has the highest frequency of LOH in many carcinomas, particularly lung cancer, and has been the target of homozygous deletions in cell lines and uncultured tumors. Importantly, the involvement of this region has been repeatedly suggested by LOH studies in breast cancer. Secondly, functional studies by Killary et al., (Killary et al., 1992) have shown that DNA segments from this region will suppress tumorigenicity of the mouse fibrosarcoma cell line, A9.

Lerman et al., (Wei et al., 1996) isolated a contiguous set of cosmid and P1 clones which span over 600 kb of this region. These clones have been partially sequenced by the Washington University Genome Center and the results have been posted on the Internet. We described in our last report how the many individual sequences were assembled and analyzed for likely genes (Fig. 7, below). BLAST homology searches revealed identities to nine known genes (black boxes) whose relative positions are shown below. Further, a number of essentially perfect matches to unannotated cDNA sequences were found, many of which coincided with strongly predicted exons.



6.C.2. Analysis by PCR for 3p21.31 Homozygous Deletions in Breast Carcinomas.

In our last report, we described how we systematically searched within the 3p21.31 region for genes whose expression is altered or extinguished in breast tumors. We have now completed a detailed search for homozygous deletions in breast cancer lines using the polymerase chain reaction (PCR). PCR analyses were used to assess the breast carcinoma cell lines for homozygous deletions in the 3p21.31 area that corresponds to three homozygous deletions identified in the SCLC cell lines NCI-H740, -H1450 and GLC-20 (Daly et al., 1993; Roche et al., 1996). PCR reactions were performed using a touch down procedure in which the primer annealing reaction begins at high temperatures (eg., 65°C) and is progressively lowered by 0.5 degrees to the optimum temperature (eg., 55°C) during the first 20 PCR cycles. The final 15 cycles are then performed at the optimum temperature. This procedure yields highly specific amplification products with a minimum of false background bands. Each reaction was performed using 40 nanograms (ng) of cell line DNA in a 20 μl reaction volume. In addition, some markers were amplified concurrently (ie., multiplexed) so that absence of a product could not be attributable to a failed PCR reaction. PCR results were obtained for 12 breast carcinoma cell lines; CRL-1504, HTB-122, HTB-23, HTB-121, HTB-123, HTB-127, HTB-131, HTB-132, MCF-7, MDA-231, T-47-D, and ZR-75.1, using 11 markers from within the 3p21.31 deletion region. The eleven markers utilized were PL6, N24898, LuCa2, IFN-rel, semaV, GNAI2, G17, transducin, semaIV, LuCa15, H29465, listed in their order along the chromosome from proximal to distal. Control amplifications included DNA from the normal human cell line FS and a reaction with no template to control for contamination. All the cell lines had amplifiable products from each marker used in the PCR amplification reaction. We found no evidence of homozygous deletions with this set of cell lines and markers.

6.C.3. Summary and Significance of 3p21.31 Findings

Despite the absence of homozyogous deletions, the genes found within this region may still be important in breast cancer; homozygous deletions represent only one of several mechanisms that generate the loss of a gene's function. Therefore, we are exploring two genes in this region until we have proof that neither is involved in breast cancer. The first is H.SemaIV, described in detail in our last report, and the second is a new gene we call hDEF-3 whose analysis has just begun. DEF-3 is a novel sequence which has properties suggestive of an RNA binding protein. We are generating expression constructs for selected portions of DEF-3 to prove that it binds RNA and to identify its binding targets. We plan on examining both H.SemaIV and hDEF-3 for mutations and for alterations both at the mRNA level and at the protein level. We believe that H.SemaIV may have a role in metastasis although we have not observed any alterations of this gene so far. As

discussed in the following section, we have actually identified two homozygous deletion regions in 3p21. Which, if any or both, is involved in breast cancer remains to be determined. Our experiments should clarify this important question. These experiments to date address primarily aims 2 and 3 for this region.

6.D. Involvement of 3p21.33 Region in Breast Carcinoma

6.D.1. Development of STS and Polymorphic Markers in Deletion Region.

Chromosomal band 3p21 harbors a second homozygous deletion region, originally described by Yamakawa et al., (Yamakawa et al., 1993), who showed that the region was missing in 5/36 SCLC cell lines. They went on to show that the minimal deletion spanned approximately 800 kb and was covered by YAC 936C1. We demonstrated that the deletion target was separated from the H.SemaIV deletion site by 10 to 15 megabases of DNA (Roche et al., 1996). In the course of mapping this region more thoroughly, we discovered that one of our markers, Mbo16E2, was present in this YAC and that it detected homozygous deletions in two uncultured lung tumors (Roche et al., 1996). This represented the first documented 3p homozygous deletion occurring in a direct tumor, and given the frequent LOH involving 3p21 should now represent a high priority region.

In our last report, we described how a lambda phage library was constructed from YAC 936C1 which was then used for sample sequencing. Our plan was to use these sequences to generate STS markers for PCR amplification and detection of homozygous deletions in breast tumor DNAs. However, we discovered that the 936C1 YAC was itself chimeric (a mixture of chromosome 3 sequences together with sequences from other chromosomes) which made most of the STS markers useless for our purposes. Instead, we have now generated an independent clone contig using P1 artificial chromosomes (PACs). This contig covers at least 750 kb and will be used to generate more STS markers as we have done before. We will also develop a number of polymorphic markers from this library and apply these to breast tumor cell line DNAs and direct tumors. Dr. Siggurdur Ingvarsson (Dept. Pathology and Cell Biology, University of Iceland, Reykjavik) has agreed to analyse a number of these novel polymorphic markers on his collection of breast tumor samples.

6.D.2. Potential Genes.

A number of cDNAs have already been identified which correctly map into the homozygous deletion region. These cDNAs have been ordered from the Research Genetics repository and primer pairs have been made to amplify them. Thus several genes may have already been identified from within the minimal 3p21.33 deletion region which can be directly tested for expression in breast tumors and cell lines.

6.D.3. Significance

Based on our observed homozygous deletions in direct tumors, as opposed to cell lines, this region seems very likely to encode a tumor suppressor gene. The reagents under development are designed to rapidly analyze breast carcinoma cell lines for deletions and uncultured tumors for deletions and LOH. This latter group of samples will be more problematic and may require microdissection of tumor cells to eliminate as much of the contaminating stromal material (with normal genomes) as possible. We are developing primer pairs for each of the cDNAs identified so far that should discriminate between genomic DNA and messenger RNA. Any of these putative genes which appears to be expressed in control tissues and is either not expressed or shows an altered product in breast tumors will be investigated further for mutations. These studies address aims 1 through 3.

7. Conclusions

In each of the previous sections comprising the Body of this report, we have provided a summary of our important results and their significance. Our studies continue to explore several distinct homozygous deletion regions on 3p. For 3p14, we have identified several additional genes which may be the target of deletions and rearrangements in breast cancers. We have previously provided strong evidence that the postulated tumor suppressor gene, FHIT, is not the target of these alterations. We are also exploring the alternative hypothesis that genomic instability, perhaps mediated by p53 mutation, is responsible for the observed deletions. The reintroduction of YACs has provided the first functional evidence that tumor suppressor activity, independent of FHIT, resides in 3p14.

Similar studies are ongoing for two additional homozygous deletion regions (proximal 3p21.3 and distal 3p21.3). A second candidate gene has been identified in the proximal 3p21.3 deletion region, and we are preparing corresponding expression constructs and antibodies. For the distal 3p21.3 deletion region, the high frequency of 3p21 LOH in breast cancers combined with our detection of homozygous deletion in direct tumors, indicates that this region must be thoroughly investigated. With our development of a high fidelity PAC contig for 3p21.33 and the identification of several corresponding cDNAs, this objective is being achieved.

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9. APPENDIX.

Figure Legends:

Figure 2. Map of clone contig and deletions in 3p14.2

A. Schematic showing position of the homozygous deletion region with respect to YACs 850A6, 65E7 and 74B2. Positions of MluI and selected XhoI restriction sites are indicated. XhoI sites were not determined for YAC 850A6. Novel genes λ58, GB and HRCA1 as well as the FHIT gene are indicated in approximate position. For FHIT, the approximate positions of exons (numbered 1 through 10) are shown. HRCA1 is within FHIT intron 3, GB is in FHIT intron 4 and λ58 is within FHIT intron 5.

B. Cosmid and lambda clone contig spanning the homozygous deletion region within 3p14. Solid horizontal lines represent cosmid (c) and lambda (λ) inserts, as indicated, along with cleavage sites for EcoRI (short vertical bars), XhoI (X) and SalI (S) which were mapped within the central 170 kb. The MluI site corresponding to the site in YAC 74B2 is present in c55D12 and c84F12, although we have not determined its precise location. Positions of selected markers, breakpoints and integration sites for HPV-16 and pSV2neo are indicated across the top. The shaded bar indicates the extent of genomic sequencing accomplished so far; the scale bar indicates kilobases. Parts B and C of this figure are drawn to the same scale and positions correspond exactly between the two.

C. Homozygous deletions in tumors and normal genomic DNAs. Horizontal lines denote the extent of deletion in the indicated cell lines. Parallel lines at the ends of each deletion indicate where precise boundaries were not determined. HeLa and MDA231 contain discontinuous deletions indicated by interrupted lines.

Figure 3. Summary of sequence features observed in FRA3B.

Assembled sequence data totaling approximately 190,000 basepairs. GeneMark and GRAIL2 predicted exons are indicated by filled boxes above the line; the λ58 and GB exons are indicated. Repetitive sequences homologous of various types are indicated by different colored bars below the line. Unique features of the sequence are indicated.

Figure 4. RT-PCR analysis of the λ58 gene.

RNA isolated from the indicated sources was treated with reverse transcriptase and the resulting cDNA was used for PCR amplification using primers specific for λ58. The control primers for GapS derived from a region within 10 kb of λ58 and more centromeric. The products were separated on an agarose gel and photographed.

Figure 5. The HRCA1 gene and exon linking.

In part A is presented a diagram of the exons found within HRCA1 so far. The six exons were identified by exon trapping (black boxes) and/or GRAIL2 (open boxes) and/or GeneMark (boxes with diagonal lines). Primers 3189 and 3186 will generate a 709 bp product from genomic DNA or unprocessed RNA. Oligonucleotide F3 was used to detect bona fide products.

Panel B shows the ethidium bromide stained gel of RT-PCR products generated using HRCA1 primers 3189 and 3186 on the indicated RNAs. Only samples treated with RTase yielded products. The YAC 850A6 acts as a genomic control.

Panel C shows the result of hybridization with the HRCA1 oligonucleotide F3, which detects correctly amplified products. Note that the 709 genomic sized product hybridizes as does a 600 bp putative spliced product.

Figure 6. Analysis of growth rate for A9 and A9-74B2g transfectants.

Cell populations of parental A9 cell line and three transfected A9 cell cultures grown in DMEM with 15% foetal calf serum, 200 μg/ml G418 (neomycin) and 50 μM $ZnSO_4$. There is no significant difference in growth rates.

Figure 2. Map of clone contig and deletion region in 3p14.2

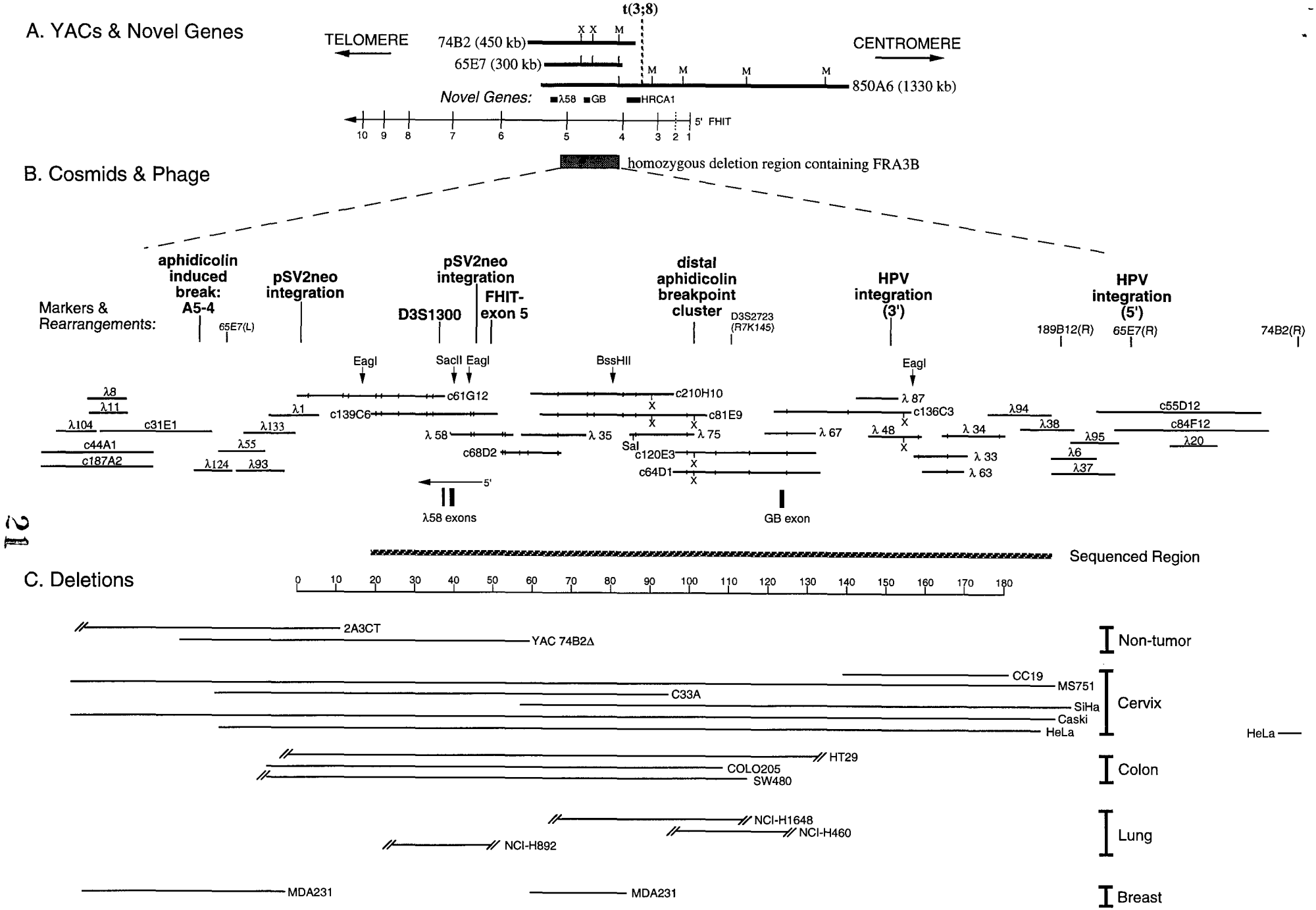


Figure 3: FRA3B Sequence Features

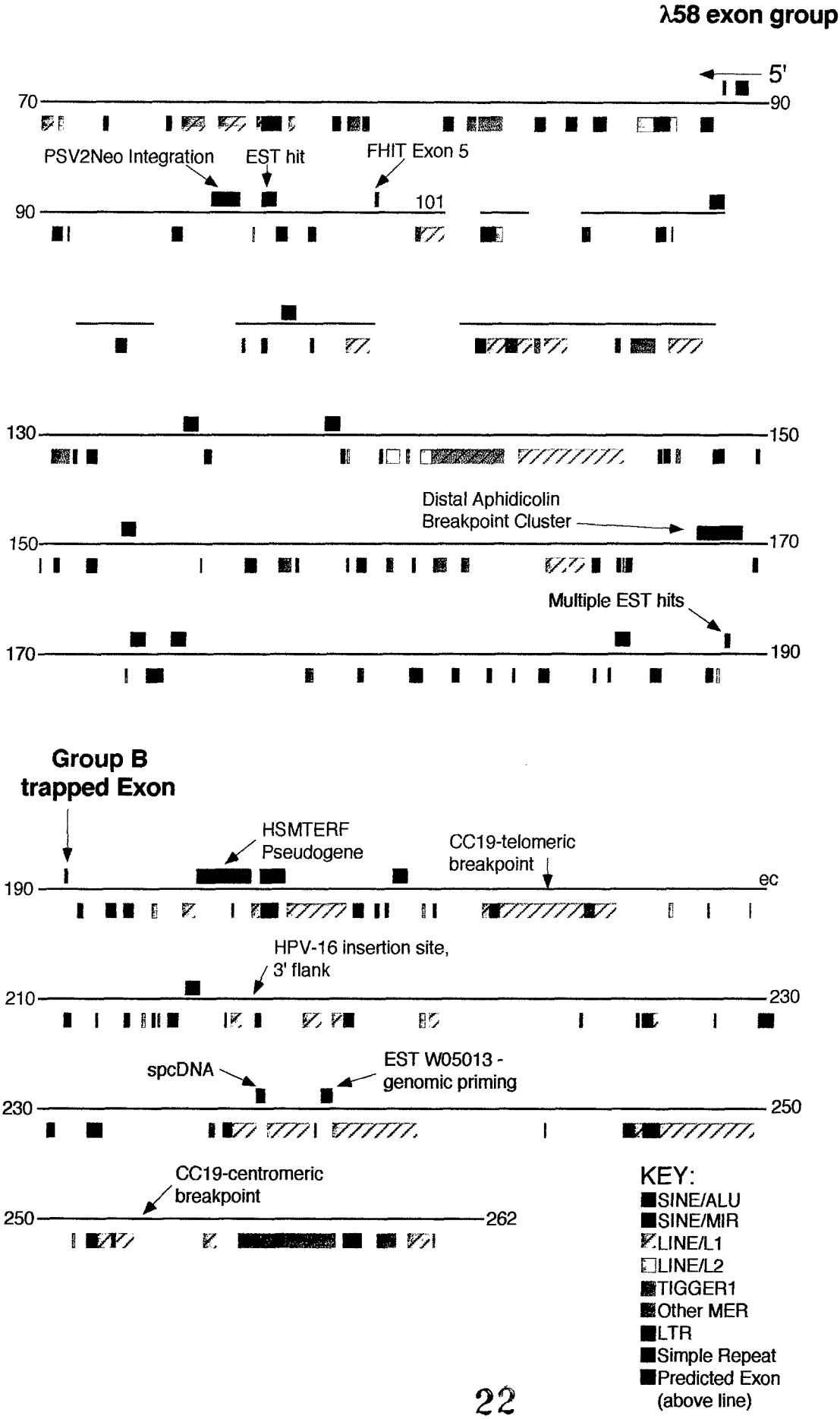


Figure 4. RT-PCR Analysis of λ58 Gene

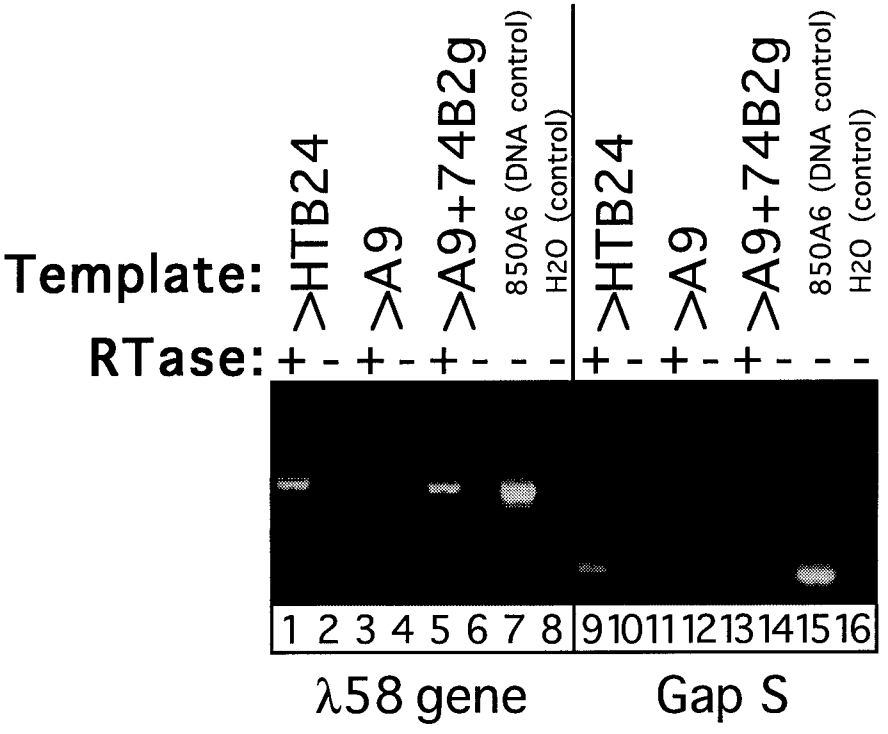
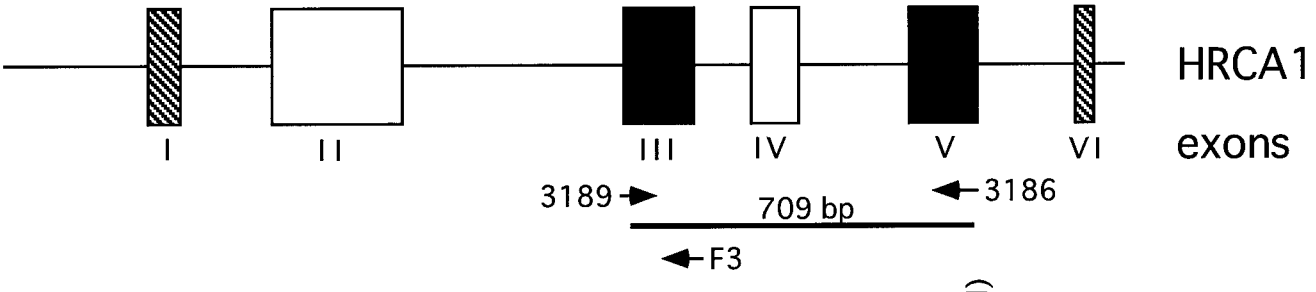
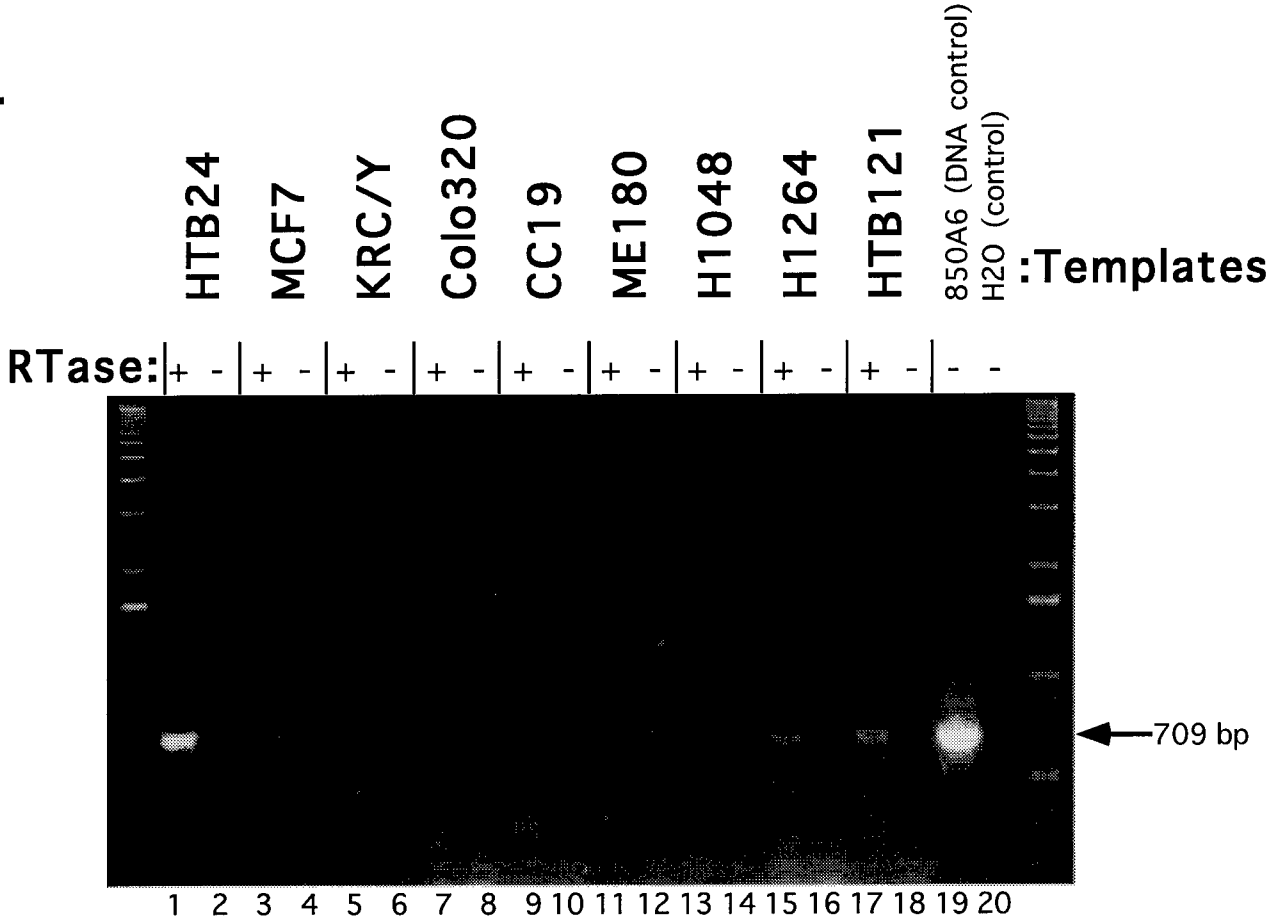


Fig. 5. RT-PCR analysis of HRCA1 expression in tumors of breast, kidney, colorectum, cervix and lung.

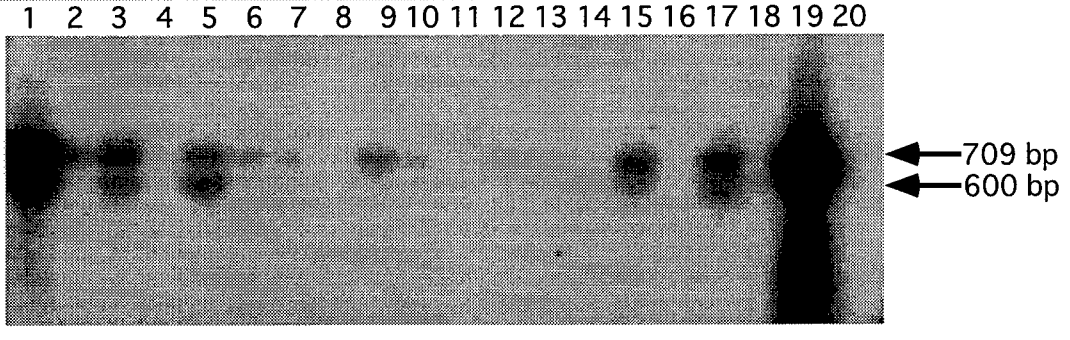
A.



B.

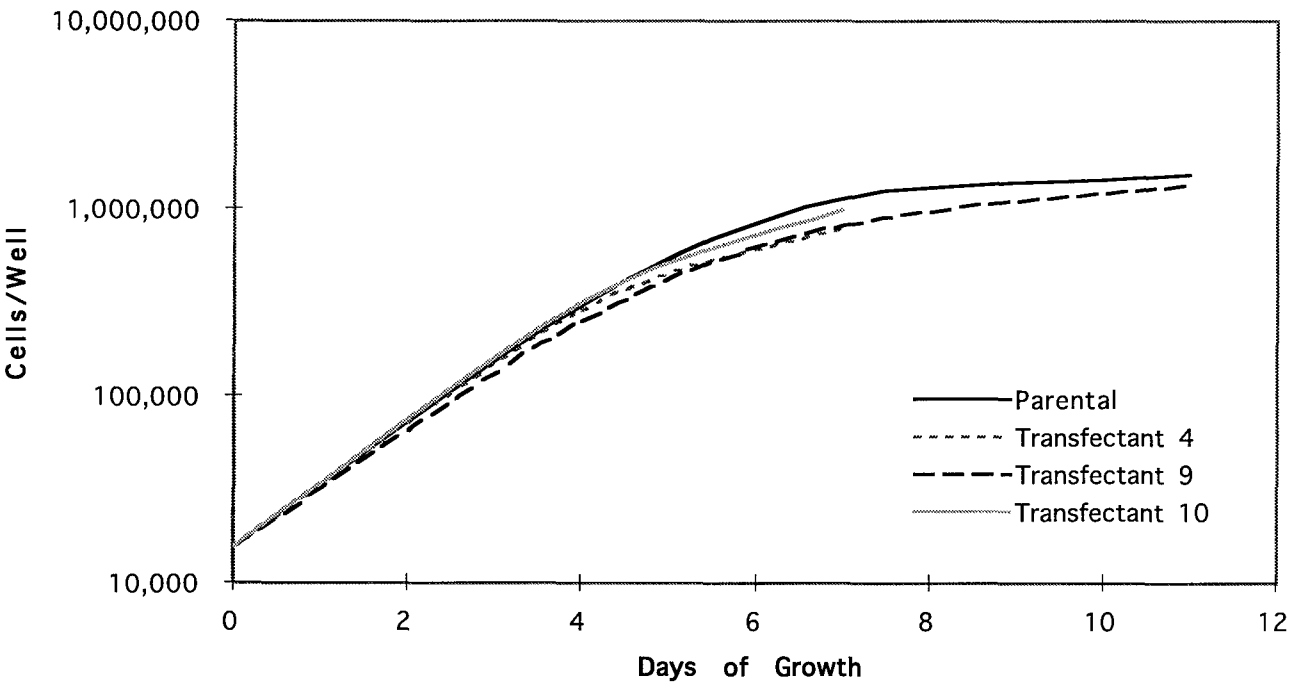


C.



Probe = HRCA1 primer F3

Figure 6: Growth properties of A9 and A9-74B2g transfectants.



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ARTICLE

Chromosome 3p14 homozygous deletions and sequence analysis of FRA3B

Ferenc Boldog[1,‡], Robert M. Gemmill[1,‡], James West[1], Misi Robinson[1], Linda Robinson[1], Efang Li[1], Joelle Roche[1,+], Sean Todd[1], Barbara Waggoner[1], Ron Lundstrom[2], Jan Jacobson[1], Michael R. Mullokandov[3], Harold Klinger[3] and Harry A. Drabkin[1,*]

[1]Division of Medical Oncology, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262, USA, [2]Eleanor Roosevelt Institute, 1899 Gaylord, Denver, CO 80206, USA and [3]Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Ave., New York, NY 10461, USA

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Loss of heterozygosity (LOH) involving 3p occurs in many carcinomas but is complicated by the identification of four distinct homozygous deletion regions. One putative target, 3p14.2, contains the common fragile site, FRA3B, a hereditary renal carcinoma-associated 3;8 translocation and the candidate tumor suppressor gene, *FHIT*. Using a ~300 kb cosmid/λ contig, we identified homozygous deletions in cervix, breast, lung and colorectal carcinoma cell lines. The smallest deletion (CC19) was shown not to involve *FHIT* coding exons and no DNA sequence alterations were present in the transcript. We also detected discontinuous deletions as well as deletions in non-tumor DNAs, suggesting that *FHIT* is not a selective target. Further, we demonstrate that some reported *FHIT* aberrations represent normal splicing variation. DNA sequence analysis of 110 kb demonstrated that the region is high in A–T content, LINEs and MER repeats, whereas Alu elements are reduced. We note an intriguing similarity in repeat sequence composition between FRA3B and a 152 kb segment from the Fragile-X region. We also identified similarity between a FRA3B segment and a small polydispersed circular DNA. In contrast to the selective loss of a tumor suppressor gene, we propose an alternative hypothesis, that some putative targets including FRA3B may undergo loss as a consequence of genomic instability. This instability is not due to DNA mismatch repair deficiency, but may correlate in part with p53 inactivation.

INTRODUCTION

LOH involving 3p occurs frequently in carcinomas of the lung, kidney, cervix, breast and other epithelial neoplasms (1–7). However, 3p loss is complex, involving at least four distinct homozygously deleted regions (8–12). One of the most frequently lost regions is 3p14, especially in cervical carcinomas (13). This region is also of interest since it contains the site of a hereditary renal carcinoma-associated translocation, t(3;8)(p14.2;q24.1) (14), and is the location of the most inducible common fragile site in the genome, FRA3B (15). We previously reported cloning of the 3;8 translocation breakpoint (16) and demonstrated by fluorescence *in situ* hybridization that FRA3B was further telomeric (17). These studies also suggested that breaks in FRA3B occur over a region instead of at a single site. While searching for genes in this region, we identified a homozygous deletion in the cervical carcinoma cell line, HeLa, involving marker *D3S1300*.

We developed a ~300 kb cosmid/λ contig within FRA3B containing *D3S1300*. Probes from the region detected frequent homozygous deletions in cervical, lung, colorectal and breast carcinoma cell lines. Cervical carcinomas, which are associated with papilloma virus infection and p53 inactivation (18), were most frequently deleted and the smallest deletion occurred in cell line CC19. During our investigations, Ohta *et al.* reported identification of a candidate tumor suppressor gene, *FHIT*, which spanned the t(3;8) breakpoint and was deleted in various carcinoma cell lines (19). However, our analysis indicates that *FHIT* is not the target of these deletions. We also observed that 3p14 deletions tend not to occur in tumors with deficiencies in DNA mismatch repair.

*To whom correspondence should be addressed
+Present address: CNRS URA1172, IBMIG, Universite de Poitiers, 40 Av. du Recteur Pineau, 86022 Poitiers Cédex, France
‡These authors contributed equally to this work

Table 1. Results of microsatellite analysis and 3p14 deletions in selected cell lines

Cell line	Mismatch repair deficiency/ microsatellite instability[a]	3p14 status	Reference
Colorectal carcinoma[b]			
HT-29	intact	deletion	this study
SW-480	intact	deletion	Umar *et al.* (25); this study
SW-403	intact	deletion	this study
SW-948	intact	rearranged	thisstudy
SW-48	defective	no deletion	Boyer *et al.* (23); this study
DLD1/HCT15	defective	no deletion	da Costa *et al.* (24)
		Bhattacharyya *et al.* (22)	
Cervical carcinoma[c]			
C-41	intact	deletion	Larson *et al.* (26)[d]
Caski	intact	deletion	Larson *et al.* (26)[d]
SIHA	intact	deletion	Larson *et al.* (26)[d]
HeLa	intact	deletion	Boyer *et al.* (23); Umar *et al.* (25)
CC19	NT	deletion	
MS-751	intact	deletion	Larson *et al.* (26)[d]
C-33A	defective	deletion	Larson *et al.* (26)[d]; this study
ME-180	intact	no deletion	Larson *et al.* (26)[d]

[a]Defective refers to a reported deficiency in mismatch repair or microsatellite instability.
[b]Overall, deletions (5) or rearrangements (1) were identified in 6/12 colorectal carcinoma DNAs. In addition to those listed, deletions occurred in COLO-205 and COLO-320. No deletions were identified in SKCO-1, CaCo-2, SW-1417 and T-84.
[c]Represents all cervical carcinoma samples examined.
[d]Personal communication from Dr Garret M. Hampton.
NT = not tested.

DNA sequencing studies were performed to identify features that might provide insight into these breaks. Our results demonstrate that FRA3B is high in A–T content, LINES and MER repeats. In contrast, Alu elements and confirmed genes are reduced. We identified a FRA3B segment highly similar to a reported small polydispersed circular DNA, sequences which are markedly elevated in damaged or unstable genomes. FRA3B also bears an intriguing overall sequence similarity to the Fragile-X region. However, unlike the rare folate-sensitive fragile sites, no triplet repeats nor methylated CpG island was identified. These overall features may be responsible for, or contribute to, the observed instability of this region.

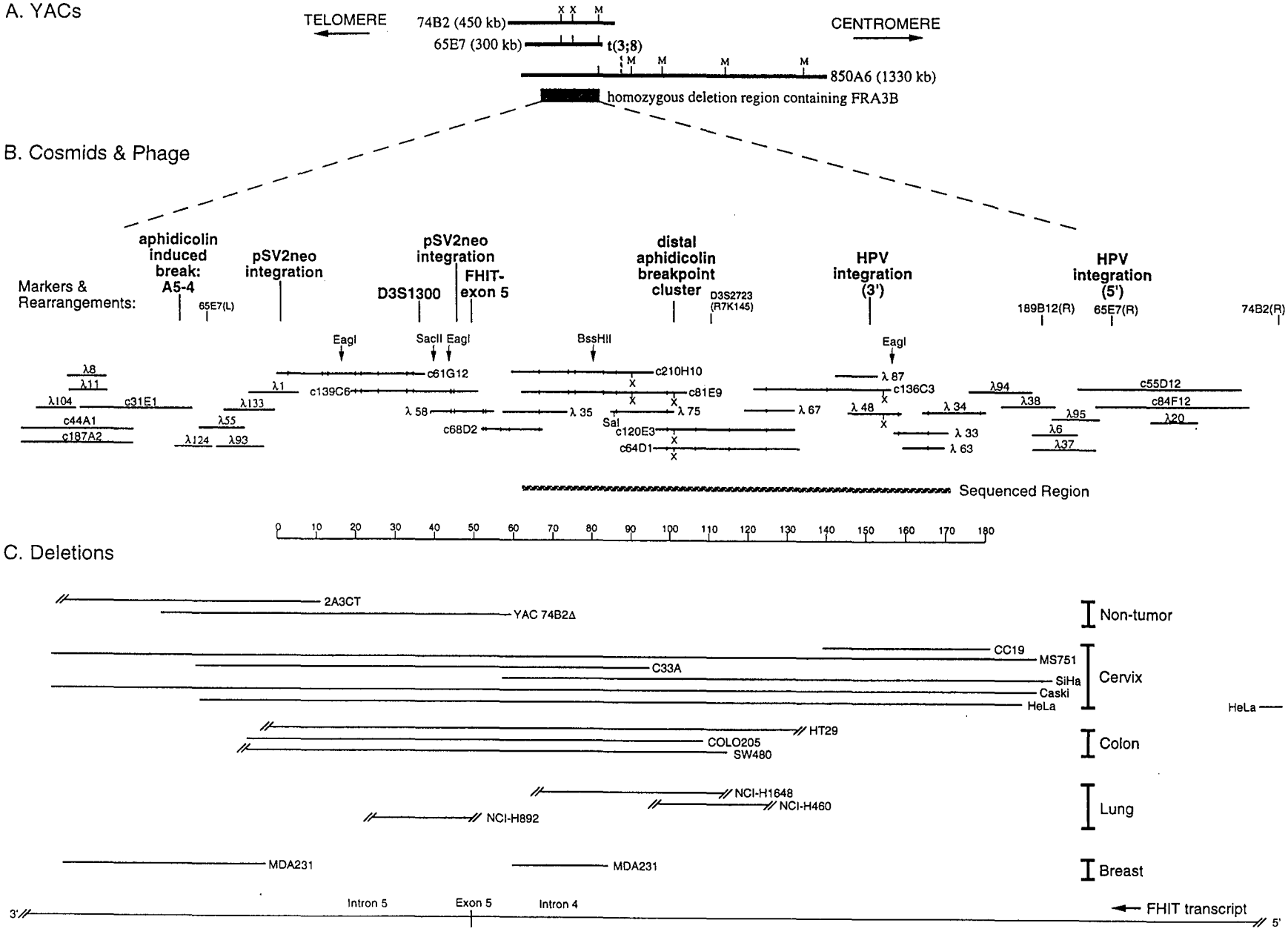
RESULTS

Identification of homozygous deletion

Following identification of the HeLa deletion, additional cervical carcinoma lines were examined using probes spanning a ~300 kb cosmid/phage contig from the FRA3B region (Fig. 1A and B). Figure 2A shows an example using cosmid c136C3. Deletions were evident in DNAs from MS751, SIHA and Caski (lanes 2, 5 and 7) whereas CC19 (lane 6) shows missing and altered bands (arrow). Altogether, homozygous deletions were detected in seven out of eight lines (87.5%). Similar hybridizations were performed using DNAs from 12 colon tumor lines, and deletions or rearrangements were seen in 50% (Fig. 2B). Other homozygous deletions (Fig. 1C) were detected in three lung (NCI-H1648, NCI-H460 and NCI-H892) and one breast carcinoma (MDA-231) cell lines. The smallest (~40 kb) was in the cervical carcinoma line CC19. This line was characterized further by DNA sequence analysis and *FHIT* gene expression (see below). Two (HeLa and MDA-231) contained discontinuous deletions, which was surprising since a single deletion should have been sufficient to inactivate a tumor suppressor gene. No deletions were detected in five renal carcinoma lines (KRC/Y, CAKI-1, CAKI-2, ACHN and KV6).

We also observed deletions in non-tumor-derived DNAs. Hybridization with c61G12 to a chromosome 3 hybrid panel revealed a partial deletion in 2A3CT (Fig. 3A). Similarly, a

Figure 1. (**A**) Schematic showing position of the homozygous deletion region with respect to YACs 850A6, 65E7 and 74B2. Positions of *Mlu*I and selected *Xho*I restriction sites are indicated. *Xho*I sites were not determined for YAC 850A6. (**B**) Cosmid and λ clone contig spanning the homozygous deletion region within 3p14. Solid horizontal lines represent cosmid (c) and lambda (λ) inserts, as indicated, along with cleavage sites for *Eco*RI (short vertical bars), *Xho*I (X) and *Sal*I (S) which were mapped within the central 170 kb. The *Mlu*I site corresponding to the site in YAC 74B2 is present in c55D12 and c84F12, although we have not determined its precise location. Positions of selected markers, breakpoints and integration sites for HPV-16 and pSV2neo are indicated across the top. (B) and (C) are drawn to the same scale and positions correspond exactly between the two. (**C**) Homozygous deletions in tumors and normal genomic DNAs. Horizontal lines denote the extent of deletion in the indicated cell lines. Parallel lines at the ends of each deletion indicate where precise boundaries were not determined. HeLa and MDA231 contain discontinuous deletions indicated by interrupted lines. Exon 5 and the direction of transcription for the *FHIT* gene are indicated at the bottom.


A. YACs
TELOMERE
CENTROMERE
74B2 (450 kb)
65E7 (300 kb)
t(3;8)
850A6 (1330 kb)
homozygous deletion region containing FRA3B
B. Cosmids & Phage
Markers & Rearrangements:
aphidicolin induced break: A5-4
65E7(L)
pSV2neo integration
D3S1300
pSV2neo integration
FHIT-exon 5
distal aphidicolin breakpoint cluster
D3S2723 (R7K145)
HPV integration (3')
HPV integration (5')
189B12(R)
65E7(R)
74B2(R)
EagI
SacII EagI
BssHII
EagI
λ8
λ11
λ104
c31E1
c44A1
c187A2
λ1
c139C6
c61G12
λ133
λ55
λ124
λ93
λ 58
c68D2
c210H10
c81E9
λ 35
Sal
c120E3
c64D1
λ 75
λ 67
λ 87
c136C3
λ 48
λ 34
λ 33
λ 63
λ94
λ38
λ95
λ6
λ37
c55D12
c84F12
λ20
Sequenced Region
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
C. Deletions
2A3CT
YAC 74B2Δ
CC19
MS751
C33A
SiHa
Caski
HeLa
HT29
COLO205
SW480
NCI-H1648
NCI-H460
NCI-H892
MDA231
MDA231
Non-tumor
Cervix
Colon
Lung
Breast
HeLa
Intron 5
Exon 5
Intron 4
FHIT transcript
3'
5'

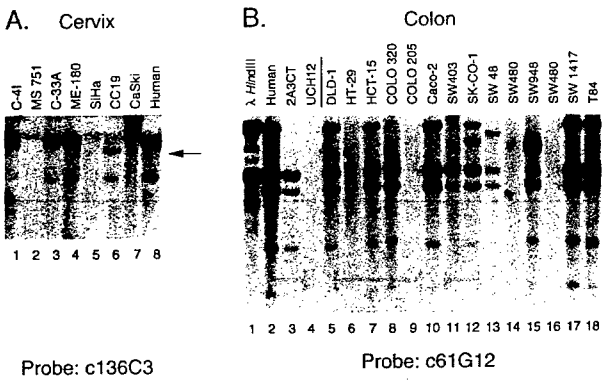


Figure 2. (**A**) Southern analysis of cervical carcinoma cell lines. Lanes 1–7 contain DNA from seven cervical lines, as indicated; lane 8 is a normal human DNA control. DNA samples were digested with *Eco*RI and hybridized with c136C3. Three cell lines were homozygously deleted for c136C3 while the arrow indicates a rearranged band present in CC19. (**B**) Southern analysis of colon carcinoma cell lines. Control lanes (1–4) contain respectively, λ *Hin*dIII size marker, *Hin*dIII-digested human DNA, the chromosome 3-specific hybrids 2A3CT and UCH12. Hybrid 2A3CT contains a single copy of chromosome 3 deleted for all sequences distal to 3p21.3. The chromosome retained in UCH12 is deleted for the entire short (p) arm. Lanes 5–18 contain *Hin*dIII-digested DNAs from 12 colon carcinoma cell lines (note, DLD-1 and HCT-15 are identical). The Southern blot was hybridized with c61G12 which contains *D3S1300* and *FHIT* exon 5. Homozygous deletions were observed in three cell lines; altered bands, which may be indicative of rearrangements, were present in four additional lines.

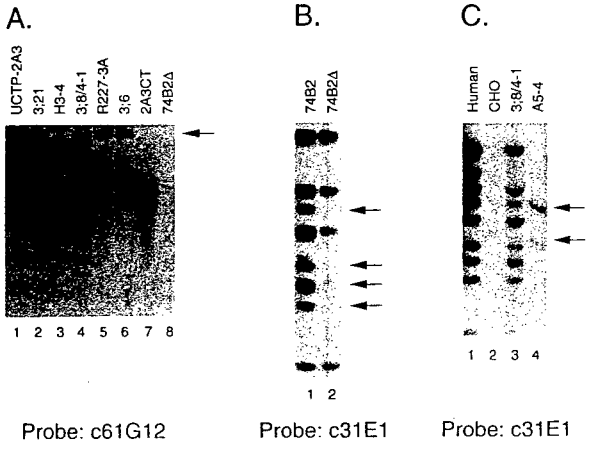


Figure 3. Deletions in genomic DNAs from normal sources. DNA from several hybrids and YAC clones were analyzed with probes from the homozygous deletion region. In (**A**), DNA from seven chromosome 3 hybrids and a deletion variant of YAC clone 74B2 were digested with *Hin*dIII and hybridized with c61G12. The variant band in hybrid H3-4 represents a polymorphism identified in eight of 19 normal DNA samples (not shown). The seven hybrids all retain 3p14.2 by cytogenetic and molecular genetic analyses while missing other specific regions of chromosome 3. (**B**) DNA samples from YACs 74B2 and the deletion variant 74B2D were digested with *Eco*RI and hybridized with c31E1. Four homologous bands present in YAC 74B2 were missing in 74B2D (arrows). In (**C**), *Eco*RI-digested DNA from the hybrid A5-4 (lane 4) was compared with human (lane 1) and hybrid 3;8/4-1 (lane 3) using c31E1.

spontaneous 80 kb deletion in YAC 74B2 spanning the region was identified during single clone purification (74B2D). Figure 3B shows missing bands (arrows) in 74B2D when hybridized with c31E1. This 80 kb deleted segment encompasses the aphidicolin-induced breakpoint in hybrid A5-4 (Figs 3C and 1C), the ends of several tumor deletions (C33A, SIHA, HeLa, COLO-205 and MDA-231) and the more telomeric of two pSV2neo plasmid insertion sites which preferentially integrated into FRA3B after aphidicolin treatment (20,21).

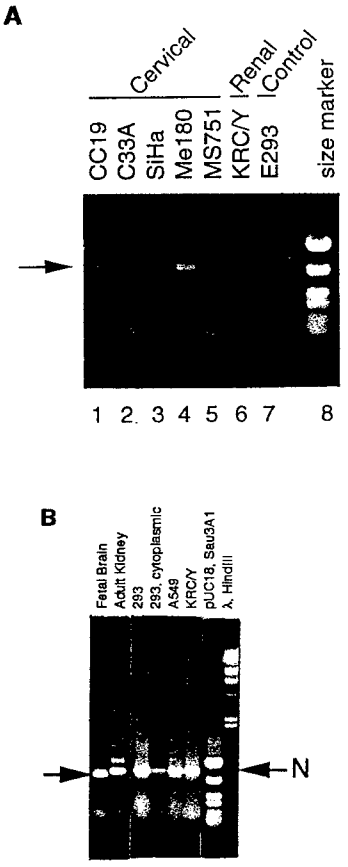


Figure 4. (**A**) RT-PCR analysis of *FHIT* expression in tumor cell lines. Primers designed to amplify the coding region of *FHIT* were used to amplify cDNAs prepared from five cervical carcinoma lines, one renal carcinoma line (KRC/Y) and an E1A-transformed embryonal kidney line (E293), as indicated. Normal products of 638 bp (arrow) were observed in three cell lines, including CC19 with a 40 kb homozygous deletion. A normal sized product was also observed in MS751, although it is amplified weakly. In (**B**), evidence is presented for alternative splicing of *FHIT* mRNA in normal tissues. RT-PCR analysis of *FHIT* expression in fetal brain, adult kidney, E293 cells (whole cells and cytoplasmic fraction), lung tumor A549 and kidney carcinoma cell line KRC/Y were performed as in (A). Both normal sized products (arrow marked N) and smaller products (left arrow) were observed.

Correlation of deletions with microsatellite instability

In cervical and colorectal carcinoma cell lines, we observed a trend of inverse correlation between the presence of a 3p14 deletion and reported microsatellite instability (22–26). However, for some lines, we were unable to discern the replication error (RER) status from the literature. Therefore, we subcloned selected lines and tested 10 clones each with up to six microsatellite loci (*D3S1300*, *D3S1210*, *D3S1286*, *D3S1233* and *AFM320yb5*). Instability was accepted if new bands appeared from two or more loci, although there were no cases of only one alteration. Results for samples where we have information on both DNA mismatch repair and 3p14 deletion are shown in

Table 1. While the number of RER+ lines is small, both 3p14 deletions and the RER phenotype are usually discordant. One simple conclusion is that defects associated with microsatellite instability alone are not responsible for the observed deletions. We also observed the highest incidence of deletions in cervical carcinoma lines, where p53 alterations appear very common (27,28). This may relate to the reduced frequency of deletions observed in RER+ colorectal tumors (see Discussion).

Relative position of *FHIT*

To examine the role of the *FHIT* gene in these deletions, we derived primers (FHIT-5′ and FHIT-3′) for a one-stage RT-PCR amplification of the entire coding region (exons 5–9). An expected 638 bp product was amplified after 35 cycles from embryonal kidney 293 cells (Fig. 4A, lane 7). This was used as a probe against the contig which showed that only a single *FHIT* exon, located adjacent to *D3S1300*, was present (Fig. 1B). Comparison of our map with that described by Ohta *et al.* (19) allowed us to conclude that this was exon 5 (also confirmed by DNA sequencing, not shown) and demonstrated that several tumor deletions exclusively affected large introns (Fig. 1C). This occurred in CC19 and SIHA (cervix), NCI-H1648 and NCI-H460 (lung) and MDA-231 (breast).

Analysis of *FHIT* in the tumor lines shown in Figure 4A demonstrated that 3/6, including CC19, contained normal sized bands (arrow). To characterize further the *FHIT* product from CC19, four independent isolates were sequenced. Each contained only normal sequences comprising coding exons 5–9. Thus, the homozygous deletion had no apparent effect on *FHIT* mRNA, suggesting either a different target gene or unselected genomic instability. Additionally, a faint normal sized product was seen in MS751 (Fig. 4A, lane 5), which by Southern blot contains a homozygous deletion including exon 5. A possible explanation is that the deletion is heterogeneous within the cell population suggesting it occurred during culture.

The cervical carcinoma lines C33A and SIHA contained multiple smaller RT-PCR products with no detectable wild-type product. While C33A had an exon 5 deletion that could explain one smaller band (Fig. 1C), the multiplicity of products suggested alternative splicing. The SIHA deletion does not affect an exon although there may be non-recognized discontinuous or overlapping bi-allelic deletions. Despite these obvious differences, the RT-PCR products appeared identical (Fig. 4A, lanes 2 and 3). To examine this, we amplified *FHIT* coding exons from RNA or cDNAs prepared from normal (fetal brain, adult kidney), immortalized (E293) and tumor (A549, KRC/Y) samples (Fig. 4B). While normal *FHIT* (arrow marked N) was observed in each, a smaller product was also seen which predominated in the normal fetal brain cDNA library. When this was cloned and sequenced, exon 8 containing the conserved histidine triad motif and possible zinc-binding site (29) was missing (not shown). This variation had been reported to represent an aberration in squamous cell carcinomas of the head and neck (30). Additional larger products were identified from the adult kidney cDNA library although these have not been characterized. Thus, alternative splicing definitely occurs in normal tissues.

DNA sequence analysis

Since CC19 contained the smallest deletion, we initiated large-scale DNA sequencing studies to identify new genes or structural features that might be responsible for the genomic instability. Six genome equivalents of sequence were obtained from c81E9, c120E3, c136C3 and λ33 and the data assembled. Gaps were closed using directed primers and a 1.3 kb clone gap between c136C3 and λ33 was closed by PCR amplification using λ48 (Fig. 1B) as template. This resulted in 110.4 kb of contiguous sequence (GenBank U66722) at an accuracy of ~99.6%. Predicted restriction maps from the assembled sequence also matched those determined experimentally. Sequence analysis (shown schematically in Fig. 5) included similarity searches, repetitive sequence identification and exon prediction.

Gene search

Overall, the sequence is AT-rich (61.1%) and very depleted in CG dinucleotides. No identities to known genes were seen. GRAIL2 predicted 15 exons of which six had moderate (<0.4), five had good (0.4–0.6) and four had excellent (>0.6) scores. Four putative exons were coincident with repeat sequences and two others (positions 8.0 and 71.5 kb) were coincident with Genemark predicted coding segments. Genemark (31) was utilized in order to implement a matrix for higher A–T content regions. While 61 potential coding segments were identified, no significant similarities were observed. Many predicted coding segments clearly occurred within LINE and MER elements, and others not directly within repeats nevertheless demonstrated similarity to LINEs. Four putative exons were clustered near position 107 kb, one of which showed perfect identity with a partially sequenced cDNA, EST-N70372. However, Northern analysis failed to identify a transcript from this region (data not shown) and the cDNA sequence included a portion of the LINE element at position 108 kb. Based on end sequences from the cDNA clone and insert length, the cDNA was co-linear with genomic DNA. Further, the 3′ end of the cDNA was coincident with a poly(A) tract in genomic DNA, all suggesting this represented an unprocessed transcript, or more likely resulted from false priming of contaminating DNA. BLASTN searches determined two additional regions with similarity to non-annotated cDNAs (positions 58.2 and 64.3 kb). However, neither showed similarity to known genes nor were directly superimposed on predicted exons. That these sequences were observed adjacent to, rather than superimposed on, predicted exons could be due to conservative prediction algorithms used which underestimate the extent of many exons. Alternatively, the homologies could be due to infrequent repeats. We note the presence of five remaining putative exons, denoted in Figure 5, having high probability scores and which do not overlap repeats.

Repeat sequence analysis

Analysis with Pythia (32) showed that 20.2% of the sequenced region is comprised of known repeats, a level comparable with other regions chosen for comparison (Table 2). However, in this 110.4 kb, LINE and MER elements make up the bulk of repeats. Intriguingly, the repeat composition is very similar to a 152 kb sequence from the Fragile-X region (33) and differs from arbitrarily selected segments in 3p21.3 and 4p16.3 (Table 2). In particular, λ33 (from position 96 to 110.4 kb) within the CC19 deletion is nearly identical in LINE element composition to the Fragile-X region. Similarly, both Fragile-X and FRA3B have a low level of predicted coding regions. Speculatively, these similarities may influence the observed tendency of both regions to undergo breakage. However, there are obvious differences between the two sites, notably the presence of a triplet repeat and

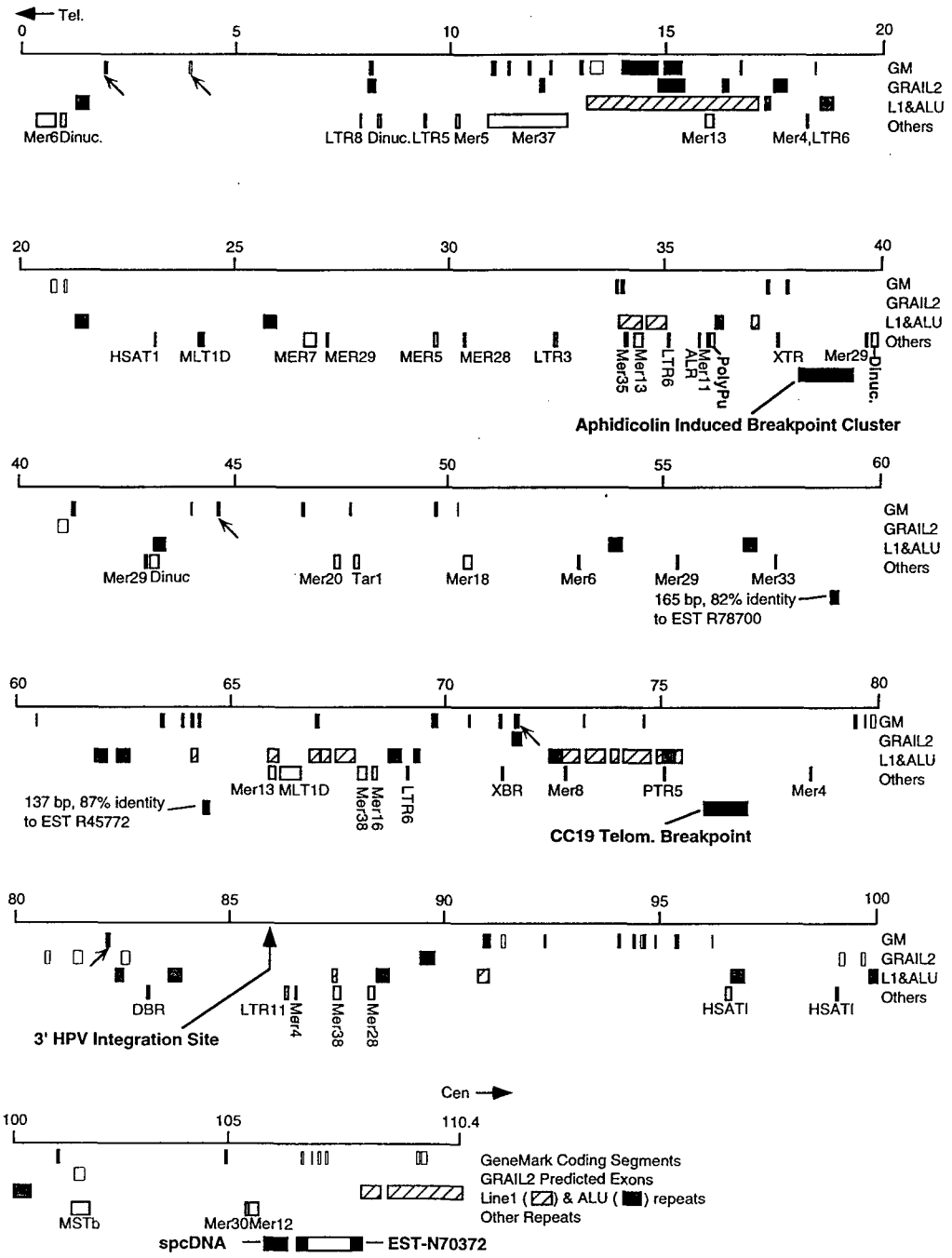


Figure 5. Assembled sequence data totaling 110 435 bp. GeneMark and GRAIL2 predicted exons are indicated (filled boxes = direct strand; open boxes = complementary strand). Repetitive sequences homologous to LINE (L1) and Alu elements are designated by boxes filled with diagonal lines and shading, respectively. Other repetitive elements are labeled. These largely consist of MER elements (medium reiterated repeats) and LTRs (long terminal repeats). Information about particular repetitive elements can be found in Repbase (ftp://ncbi.nlm.nih.gov/repository/repbase). Unique features of the sequence are indicated on the lowest row.

methylated CpG island in Fragile-X (34) and the common versus rare nature of FRA3B.

Other features

Position 38 kb contains a cluster of terminal deletion breakpoints induced by aphidicolin treatment (GenBank U46001) further linking our sequence to FRA3B. Flanking these aphidicolin breakpoints is a long polypurine tract and two extended variable dinucleotide repeats. The telomeric breakpoint of the CC19 deletion lies within a 1.0 kb region at position 76 kb between L1 and MER/LTR elements. Within the CC19 deletion region, near position 86 kb, is an HPV16 integration site from a cervical carcinoma (35). It is of note that this integration was associated with an interstitial deletion (35,36) which we now know would not affect *FHIT* coding sequences. Interestingly, a very signifi-

cant similarity ($1e^{-77}$) to a small polydispersed circular (spc) DNA was observed at 106 kb (GenBank X96885). Figure 6 shows a FASTA alignment over 585 bp. The region is 72.1% identical and contains interspersed blocks having up to 89% identity (e.g., spcDNA bp 121–207). This spcDNA, which appears non-repetitive, was isolated from a tuberous sclerosis-associated angiofibroma (I. Hinkel-Schreiner, Ph.D. Thesis). Characteristics of spcDNAs include derivation from chromosomal sequences (37), association with clustered repeats [such as β-satellites and other clustered elements although a single family member may be predominantly involved (38)] and elevation in conditions associated with genomic instability such as Fanconi's anemia (39). spcDNAs are also increased by DNA-damaging agents (37) and inhibitors of DNA and protein synthesis including the fragile site inducer aphidicolin (40). Given the limited number of spcDNAs that have been sequenced, this similarity may be biologically important.

DISCUSSION

Using a ~300 kb cosmid/λ contig, located ~150 kb telomeric to the 3;8 translocation breakpoint, we have identified homozygous deletions in various carcinoma cell lines that overlap the most inducible common fragile site in the genome, FRA3B. From various aphidicolin-induced breakpoints and plasmid or viral integration sites (Fig. 1), FRA3B represents a region rather than a single site. Studies by Wilke *et al.* (36) and Smith *et al.* (41) indicate that some clustering of breakpoints may occur. However, their rearrangements were induced by aphidicolin in a single chromosome 3-containing hybrid and, unlike the interstitial deletions we observed in tumor and non-tumor samples, appear to represent terminal breaks. Where we have defined the boundaries for the carcinoma-associated deletions accurately, one or both are contained within the FRA3B region.

Our initial hypothesis was that the smallest deletion (CC19) would contain elements of a tumor suppressor gene. During our studies, Ohta *et al.* (19) identified the *FHIT* gene with reported abnormalities in RT-PCR products. However, *FHIT* has similarity to a yeast di-adenosine hydrolase which would represent an unexpected function for a tumor suppressor gene. Our results indicate that *FHIT* is not the target of these deletions. First, the CC19 deletion does not involve *FHIT* coding sequences (Fig. 1B and C) and, based on RT-PCR and cDNA sequence analysis, the coding portion of the *FHIT* transcript is normal. Second, we have observed deletions in genomic DNAs from non-tumor sources (Fig. 3A). The somatic cell hybrid 2A3CT was formed by spontaneous terminal deletion at 3p21.3 from a non-tumor-derived chromosome 3 hybrid, UCTP2A3 (11). However, 2A3CT also acquired a 3p14 interstitial deletion overlapping an aphidicolin-induced breakpoint, a pSV2neo integration into FRA3B (20) and the telomeric borders of several carcinoma-associated deletions. We also identified an overlapping deletion in an unselected subclone from YAC 74B2. While neither the hybrid nor YAC 3p14 DNA segments are in a 'normal' background, they clearly are unselected from a tumorigenic standpoint. We note that 'hotspots' of recombination in human DNA can be maintained in a yeast background (42), thus it is not unreasonable that unstable regions may behave similarly or more so. Third, discontinuous deletions appear common in this region (i.e. HeLa, MDA231), both from our analysis and from that reported by Ohta *et al.* (19). Multiple deletions might be expected if there were no common target gene and if the region was unstable. Fourth, we have observed that *FHIT* undergoes alternative splicing in normal tissues (Fig. 4B) which explains some previously reported abnormal PCR products (19,30,43). Thiagalingam *et al.* (44) recently reported lack of *FHIT* involvement in colorectal carcinomas and suggested that PCR artifacts might be responsible for some previously observed alterations (19,43). It also seems likely that many deletions would have been missed in their study since only a few markers were tested. One of the possible features that suggested *FHIT* could be a tumor suppressor gene was that it crossed the hereditary renal carcinoma-associated 3;8 breakpoint (19). However, we found no alterations in RT-PCR products from five renal carcinoma cell lines. Moreover, Bugert *et al.* (manuscript submitted) have observed normal *FHIT* transcripts and no point mutations in a large series of renal cancers. Thus, *FHIT* does not appear to be involved in renal carcinoma. With respect to other possible target genes, from our sequencing studies we identified a 100% identity to two expressed sequence tags from a liver/spleen library. However, we determined that both clones were identical, were not expressed using a commercial Northern blot (Clontech), were co-linear with genomic DNA containing a poly(A) tract corresponding to their 3′ end, encoded no significant open reading frame and overlapped a partial LINE element.

While other tumor suppressor genes may exist within FRA3B, an alternative possibility is that the deletions are due to primary genomic instability affecting a particularly susceptible region. This hypothesis is consistent with several of the observations reported here including their discontinuity and occurrence in non-tumor cell lines. By using numerous probes, we were able to identify a high frequency of homozygous deletions, especially in cervical carcinomas where p53 inactivation is very common (18). In this regard, it is interesting that we observed an inverse correlation between 3p14 deletions and microsatellite instability (RER+). Importantly, p53 mutations, which have been shown to destabilize the genome (45,46), appear infrequent in RER+ colorectal carcinomas and gastric tumors [(47,48) and P. Cottu, presented at Cancer and the Cell Cycle, Lausanne, Switzerland 1996]. Thus, these findings would be consistent, at least in part, with 3p14 deletions resulting from the genomic instability which accompanies p53 inactivation. We note that although CC19 is HPV negative, it expresses a mutant p53 protein (53).

What have we learned from the DNA sequence analysis to date? First, is that the region is high in A–T content with frequent LINEs and MER repeats, and is conversely low in Alu sequences and confirmed genes. In contrast to the reported rare folate-sensitive sites which are associated with expanded CGG repeats (49,50), FRA3B does not contain an expanded triplet repeat or methylated CpG island; nor did we identify any telomeric repeats which have been suggested as a possible cause of breaks (51). However, we do note that over the 110.4 kb region there is an overall repeat sequence similarity to 152 kb from Fragile-X. *In vitro*, expanded CGG repeats have been shown to inhibit DNA replication (52). Whether or not specific sequences within FRA3B may have a similar effect on replication awaits experimental testing. Our discovery of a strong spcDNA homology in FRA3B may therefore not be coincidental. While we do not know if the spcDNA site is a primary cause of FRA3B instability or simply a marker for this property, the DNA sequences reported should provide the means to test this.

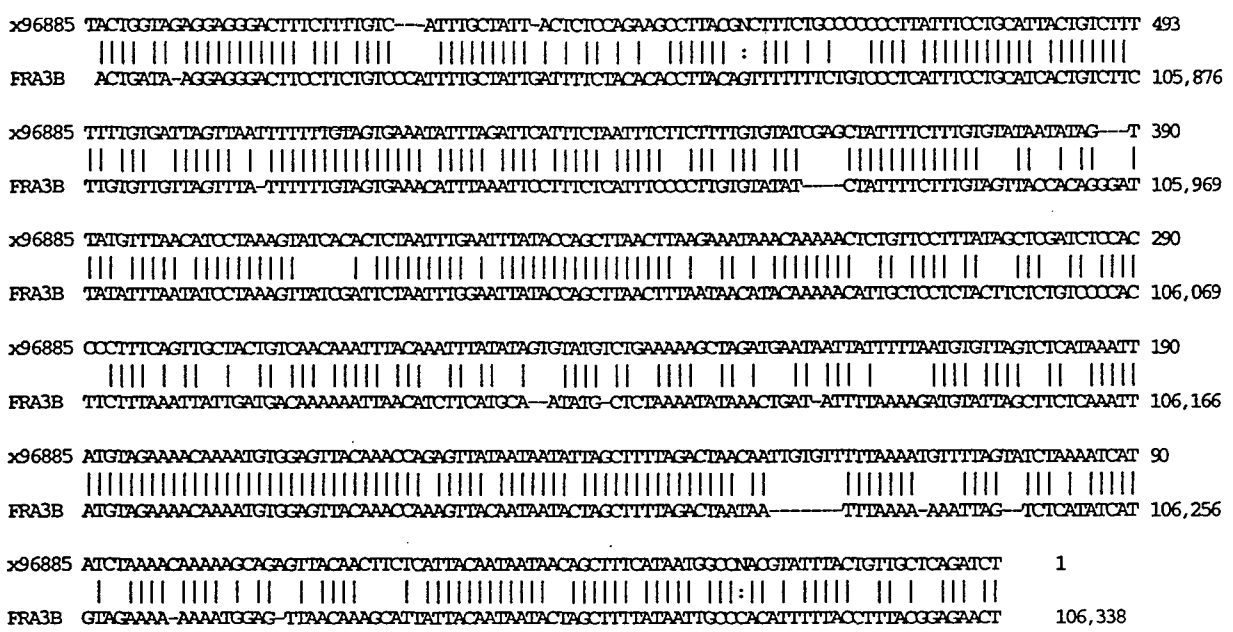


Figure 6. FASTA alignment of 585 bp of spcDNA clone (X96885) and FRA3B.

Table 2. Summary of sequence features

	Sequence length	GC content	Total repeats	Alu	LINE	MER	Predicted exons	Confirmed genes
FRA3B (110.4 kb sequence)	110 434	38.9%	20.2%	4.9%	9.0%	3.9%	3.4%	0.0%
FRA3B (λ33 only)	13 121	37.0%	26.6%	3.5%	17.5%	2.1%	2.3%	0.0%
Xq27.3 (Fragile X)	152 351	39.0%	26.3%	5.9%	16.3%	3.0%	3.4%	2.8%
3p21.3 (HLuca_14)	36 597	58.0%	19.4%	18.1%	0.0%	0.4%	9.5%	12.9%
4p16.3 (Huntington's)	32 100	54.9%	16.1%	10.8%	3.5%	1.4%	5.6%	7.6%

The sequences used for comparison were derived from Xq27.3 (contains the Fragile-X region, GenBank L29074) and 4p16.3 (in the region of Huntington's disease, GenBank Z69837). The sequence from 3p21.3 was downloaded from ftp://genome.wustl.edu/pub/gsc1/sequence/st.louis/human/shotgun/3/H_LUCA14.seq.

MATERIALS AND METHODS

Nucleic acid sources and manipulations

Cell lines. Breast, colon and cervical carcinoma lines were obtained from the American Type Culture Collection. The cervical carcinoma cell line, CC19, was established as described (53). Lung tumor lines were obtained through the Colorado Lung Cancer SPORE Tissue Culture Core Laboratory. Normal cell lines included the human lymphoblastoid cell lines TL8229 and AG4103 and the E1A-immortalized human embryonic kidney cell line, E293. The somatic cell hybrids have been described previously (54).

Libraries. The gridded chromosome 3-specific cosmid library (55) was obtained from Lawrence Livermore National Laboratories. The 850A6 YAC subclone library was previously described (16). Fetal brain and adult kidney cDNA libraries were obtained from Clontech.

DNA and RNA isolations. DNA was isolated from cell lines by standard methods. Cosmid DNAs were isolated using alkaline lysis; preparations used for DNA sequence analysis were purified further by CsCl gradient centrifugation. DNA was isolated from single or pooled phage clones by the Grossberger method (56). RNA was isolated from cell lines when the cultures reached 90% of confluency using the RNA-STAT-60 kit from Tel-Test, Inc. (Friendswood, TX).

Library screening, contig assembly and hybridization analysis. The gridded cosmid library was spotted onto filters at high density (1536 clones per filter) and hybridized using standard techniques with a 370 kb *Mlu*I fragment derived from YAC 74B2. The 850A6 YAC phage library (16) was screened using the same probe. Resulting clones were assembled into a contiguous segment by hybridization analysis using end probes and total inserts followed by analysis with the software tool SEGMAP. Complete and partial digestion analyses (57) were used to restriction map the central 170 kb of the contig prior to DNA sequence analysis.

Replication error (RER) analysis

Biotinylated primers were obtained from Research Genetics, Huntsville, AL. PCR amplifications were performed with 40 ng of template DNA utilizing hot start and touch down procedures. After separation on denaturing polyacrylamide gels, PCR products were detected using the New England BioLabs Phototope™ Detection Kit. Alleles were scored by visual inspection of band patterns.

RT-PCR analysis of *FHIT* gene expression

RT-PCR was performed using primers FHIT-5′ (5′-CTCGAATTCTTAGACCCTCTATAAAAGC-3′) and FHIT-3′ (5′-CTGATTCAGTTCCTCTTG-3′) derived from non-coding exons 4 and 10, respectively. First strand synthesis was accomplished with 1–3 mg of total RNA and the Superscript II kit (Life Technologies). Subsequent amplification utilized one-fifth of the reverse transcriptase reaction together with the *FHIT* primers. Standard PCR conditions of 94°C denaturation (1 min), 55°C annealing (1 min) and 72°C elongation for 35 cycles were employed. PCR products were subcloned into the *Eco*RI site of pBluescript II SK$^+$ using an introduced *Eco*RI site present in the FHIT-5′ primer and a natural site located 21 bp downstream of the FHIT stop codon.

DNA sequence determination

Cosmid and phage clones were sequenced using a random shotgun subcloning and end-sequencing strategy. Clone DNA was sonicated and size selected by LMP-agarose gel electrophoresis. Recovered fragments of 1–2 kb were end-repaired with Klenow fragment of *Escherichia coli* DNA polymerase I and T4 DNA polymerase, ligated into the phosphatased *Eco*RV site of pBluescript II and transformed into *E.coli* DH10B. Amp^R/β-Gal$^-$ subclones were grown in 3 ml of TB for isolation of sequencing templates. To eliminate vector sequences from the subclone library, phage inserts were amplified by long-range PCR. Inserts were gel purified, ^{32}P labeled and hybridized to subclone libraries. Positive subclones were sequenced using an ABI 373 or 377 and the ABI Prism dye terminator cycle sequencing kit. Chromatograms were transferred to a SUN workstation, analyzed and assembled using PHRED and PHRAP (from Dr Phil Green). Gaps were closed by primer walking. Based on independently obtained overlapping contigs, as well as analysis of cosmid vector sequences in some subclones, sequencing accuracy was at least 99.6%.

Sequence analysis

Assembled sequences were analyzed for database similarities by BLASTN and BLASTX, searching the nr and dbest databases with default parameters. Strongly similar sequences ($P < e^{-30}$) were retrieved from GenBank for further analysis. Homologies to repeat sequences were found using Pythia (32). Potential exons were identified using GRAIL2 (58) and GeneMark (31) programs. GeneMark was run using 5th order matrices trained on sequences with GC content similar to the sequences checked. Sequence alignments were prepared using the FASTA program from the Wisconsin Package (GCG).

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NOTE ADDED IN PROOF

Sequencing of the CC19 deletion region has been completed with the finalization of lambda 94, a phage proximal to lambda 33. This 18 810 bp sequence maintains the same characteristics as the balance of FRA3B. It is 63% AT and 27% is comprised of known repeats with the L1 type elements predominating.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

DAMD17-94-J-4413	ADB261602
DAMD17-96-1-6112	ADB233138
DAMD17-96-1-6112	ADB241664
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DAMD17-97-1-7084	ADB238008
DAMD17-97-1-7084	ADB251635
DAMD17-97-1-7084	ADB258430
DAMD17-98-1-8069	ADB259879
DAMD17-98-1-8069	ADB259953
DAMD17-97-C-7066	ADB242427
DAMD17-97-C-7066	ADB260252
DAMD17-97-1-7165	ADB249668
DAMD17-97-1-7165	ADB258879
DAMD17-97-1-7153	ADB248345
DAMD17-97-1-7153	ADB258834
DAMD17-96-1-6102	ADB240188
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DAMD17-96-1-6066	ADB235510
DAMD17-96-1-6029	ADB259877
DAMD17-96-1-6020	ADB244256
DAMD17-96-1-6023	ADB231769
DAMD17-94-J-4475	ADB258846
DAMD17-99-1-9048	ADB258562
DAMD17-99-1-9035	ADB261532
DAMD17-98-C-8029	ADB261408
DAMD17-97-1-7299	ADB258750
DAMD17-97-1-7060	ADB257715
DAMD17-97-1-7009	ADB252283
DAMD17-96-1-6152	ADB228766
DAMD17-96-1-6146	ADB253635
DAMD17-96-1-6098	ADB239338
DAMD17-94-J-4370	ADB235501
DAMD17-94-J-4360	ADB220023
DAMD17-94-J-4317	ADB222726
DAMD17-94-J-4055	ADB220035
DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754